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ERRATUM

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Para. 2 of SUMMARY should read:

2. The anti-diabetogenic activity of the serum was completely absorbed by a prolactin fraction but only partially so by growth and thyrotrophic pituitary extracts; the activity abolishing the normal glycosuria in partially depancreatized rats was absorbed by all three extracts.

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FURTHER INVESTIGATIONS ON THE MECHANISM OF OESTRONE PRODUCTION IN THE OVARY

By B. ZONDEK and J. SKLOW

From the Hormone Research Institute, Hebrew University, Jerusalem

(*Received 2 July 1941*)

Following the injection of chorionic gonadotrophin into immature female rats, oestrogen is produced in the ovary and passes into the circulation between the 26th and 27th hour after the injection. This has been shown in two experiments. First, if the injected rat is ovariectomized before the 26th hour, oestrus is inhibited, but if the ovaries are only removed 27 hr. after the gonadotrophin injection oestrus occurs normally [Zondek, 1940]. The time involved has been confirmed by experiments [Zondek, 1940; Zondek, Sulman & Sklow, 1941] in which antigonadotrophin has been injected into immature female rats at various times after injecting chorionic gonadotrophin. If the antihormone is given up to 26 hr. after the chorionic gonadotrophin the normal reactions of the rats (oestrus and the appearance of 'blood-points' and corpora lutea in the ovaries) are partially or completely inhibited, while if the antihormone is given 27 hr. after the gonadotrophin there is no inhibition.

It is possible that the oestrogen is produced in the ovary solely in the 26th hour after the injection but it is also possible that the interval between the gonadotrophin injection and the passage of the oestrogen into the circulation is occupied by the formation of an oestrogenically inactive precursor ('pro-oestrogen') in the ovaries. The experiment recorded below has been devised to test these points.

An injection of chorionic gonadotrophin into immature female rats has been followed 18 hr. later by the injection of sufficient antigonadotrophin to neutralize the response of the rats. This antihormone has been followed in its turn by a second gonadotrophin injection, 20 hr. after the first. If the oestrogen produced is formed in the ovaries only in the single hour 26 hr. after the injection of gonadotrophin, the oestrous response of the rats should occur 20 hr. later than it would after the single gonadotrophin injection. If, however, pro-oestrogen is already present in the ovary when the antihormone is injected this may be transformed into oestrogen under the influence of the second gonadotrophin injection so that the oestrous response should not be delayed. This is actually what happens.

METHODS

Chorionic gonadotrophin

The gonadotrophin used was 'Korotrin', containing 100,000 I.U./g. This was diluted with glucose so that 1 I.U. was present in 6 mg. A dose of 0·25 I.U. given to an immature rat in six subcutaneous injections produced follicle maturation and oestrus (APR I [Zondek, 1926, 1935; Zondek & Aschheim, 1927]).

Antigonadotrophin

The antigonadotrophin was prepared from the serum of a goat which had been subcutaneously injected for several months with a daily dose of 250 I.U. of chorionic gonadotrophin. The antihormone was obtained as a stable dry powder by an acetone-precipitation method [Zondek & Sulman, 1937]. Its activity was assayed by its capacity to inhibit the response of immature female rats to the simultaneous injection of chorionic gonadotrophin. One anti-unit is defined as that amount which will inhibit the activity of 1 I.U. of chorionic gonadotrophin. Our preparation contained 2·5 anti-units/mg.

Animal procedure

The experiment was performed on sixty immature female rats each weighing 30 g. The chorionic gonadotrophin and antigonadotrophin were both given in single injections. Vaginal smears were taken at 12-hr. intervals from the 60th to the 120th hour after the first injection—smears being taken as positive only when full cornification was present. At the end of the experimental period the rats were killed and their ovaries and uteri examined; the latter were swollen in all cases where a positive smear had been recorded.

RESULTS

A group of twenty-five immature rats were injected with 2·5 I.U. of chorionic gonadotrophin per rat. The times at which oestrus occurred are given in Table I.

Table I. *Time of oestrous response in immature rats injected with chorionic gonadotrophin or chorionic gonadotrophin and antigonadotrophin*

Rat nos.	No. of rats	Injections: Dose	Injections: Time	No. of rats in oestrus: 60 hr.	72 hr.	84 hr.
6482–506	25	2·5 I.U.	0 hr.	2	7	16
6507–16	10	2·5 I.U. 5·0 anti-units	0 hr. 18 hr.	0	0	0
6517–41	25	2·5 I.U. 5·0 anti-units 7·5 I.U.	0 hr. 18 hr. 20 hr.	2	5	18

A further control group was used to show that the antigonadotrophin injection given 18 hr. after the gonadotrophin had been given would inhibit the oestrous response. It is already known that chorionic gonadotrophin rapidly disappears and that 18 hr. after its injection only 15 % of the dose can be detected in the body [Zondek *et al.* 1941] so that only 0·5 anti-unit should be necessary to neutralize the action of 2·5 I.U. given 18 hr. previously. In order to be quite certain that neutralization was complete we injected 10 times this amount. A group of ten rats was used in this test and no changes occurred in their vaginal smears, ovaries or uteri.

In the main experiment twenty-five immature rats were injected with 2·5 I.U. of chorionic gonadotrophin, 18 hr. later with 5 anti-units of antigonadotrophin and 2 hr. after the latter injection with a second injection of 7·5 I.U. of chorionic gonadotrophin. Reference to the table will show that there was no appreciable delay in the onset of oestrus in this group of rats when compared with the first group.

This demonstrates that oestrogen is not produced solely during the 26th hour after the gonadotrophin injection and suggests that production of an inactive precursor does occur. It might be postulated that oestrogen is being formed throughout the 26 hours after the gonadotrophin injection but only reaches a threshold concentration at the end of this period. This is not, however, borne out by the results of the second control group of rats. Subthreshold amounts of oestrogen may be detected by a decrease in the number of leucocytes and the presence of epithelial or cornified cells in the vaginal smear—this was not found, the vaginal smears being completely negative in this group.

We therefore conclude that an inactive 'pro-oestrogen' is formed in the ovary during the 18 hr. following the injection of chorionic gonadotrophin and that this 'pro-oestrogen' is converted into oestrogen by the second gonadotrophin injection.

SUMMARY

Previous work has shown that oestrogen passes into the circulation during the 26th hour following injection of chorionic gonadotrophin into immature rats.

Oestrus may be prevented by an injection of antigonadotrophin into the rats 18 hr. after the injection of chorionic gonadotrophin, but if a second dose of chorionic gonadotrophin is injected 2 hr. after the antihormone, oestrus occurs at the same time as it would have occurred if the first injection alone had been given.

It is concluded that an inactive 'pro-oestrogen' is formed in the ovary during the 18 hours after the injection of chorionic gonadotrophin.

This work has been carried out with the aid of a grant from the Rockefeller Foundation. We are very grateful to the Winthrop Chemical Company, N.Y., for the supply of 'Korotrin'.

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CORTICOTROPHIC ACTIVITY IN PREGNANT MARES' SERUM

By Y. M. L. GOLLA and M. REISS

From the Burden Neurological Institute, Stapleton, Bristol

(*Received 8 July 1941*)

It is a well-known fact that hypophysectomy leads to a decrease in the size and weight of the adrenal glands with diminution of the lipoid content of the cortex and consequent development of a sudanophobe zone in the zona fasciculata. Administration of anterior pituitary extracts in hypophysectomized animals causes an increase in the size and weight of the adrenals, restoration of the lipoid content and disappearance of the sudanophobe zone.

Various methods have been proposed for the standardization of such pituitary corticotrophic extracts. Collip, Anderson & Thompson [1933] removed and weighed one adrenal from rats 10 days after hypophysectomy; the rats were then treated with extract and the increase in weight of the remaining adrenal was measured. They had already established that after removal of the first adrenal under these conditions there is no compensatory hypertrophy of the second, owing to the absence of the pituitary corticotrophic action. They found a mathematical relation between the dose of extract and the increase in adrenal weight. Bates, Riddle & Miller [1940] used the increase in the adrenal weight in 2-day-old chicks as the basis of their assay. Reiss, Balint, Oestreicher & Aronson [1936] found that the sudanophobe zone disappeared in hypophysectomized rats treated with pituitary extracts in doses that did not necessarily cause an increase in adrenal weight and accordingly used this test as a method for standardizing extracts. They found that the activity of extracts in causing disappearance of the sudanophobe zone was not always paralleled by the activity in causing increase in adrenal weight.

The experiments described below tend to show that there are in fact two different corticotrophic factors responsible for these two types of activity.

EXPERIMENTAL

It has been found that injections of pregnant mares' serum or serum extracts into hypophysectomized rats will prevent the further atrophy of the adrenals or lead to an increase in adrenal weight and that injections of the serum into 9-day-old chicks invariably causes an increase in adrenal weight. Some sera of other animals (rabbits, rats, etc.) have occasionally

also shown similar activity. In no single instance, however, in spite of very large dosage, has treatment of hypophysectomized rats with pregnant mares' serum caused the disappearance of the sudanophobe zone.

Table I shows the results of a series of injections of active extracts into hypophysectomized rats. The right adrenal was removed some days after hypophysectomy to serve as a control. The left adrenal was removed after the animal had been treated with pituitary corticotrophic extract, acetone-dried pregnant mares' serum or with a sample of boiled serum. It can be seen that there was an increase in adrenal weight following the injection of all three substances, though in none of the cases does the increase bear any close relation to the dose used. The sudanophobe zone disappeared only in the animals injected with the pituitary extract and in this experiment there does appear to be a relation between dose and response.

Table I. *Response of hypophysectomized rats to corticotrophic extracts injected twice daily for 7 days*

	Right adrenal before treatment				After treatment		
						Left adrenal	
Rat no.	Removal days post-op.	Weight mg.	Sudanophobe* zone	Daily dose mg.	Wt. of testes mg.	Weight mg.	Sudanophobe* zone
(*a*) *Treatment with pituitary corticotrophin*							
333	12	8·0	–	2 × 0·6	450	8·0	–
335	12	8·0	–	2 × 0·6	330	6·5	–
336	12	7·0	–	2 × 1·0	420	8·0	–
337	12	7·0	–	2 × 1·0	550	7·0	–
338	13	7·0	–	2 × 2·0	380	8·0	+
339	10	7·0	–	2 × 2·0	400	7·0	±
340	10	5·5	–	2 × 3·0	380	7·0	+
341	11	6·0	–	2 × 3·0	400	6·0	+
(*b*) *Treatment with dried pregnant mares' serum*							
239	16	3·0	–	2 × 25	...	7·0	–
226	22	5·0	–	2 × 25	...	7·0	–
225	17	5·0	–	2 × 25	...	7·0	–
247	13	4·5	–	2 × 25	840	8·0	–
252	13	4·0	–	2 × 25	560	8·0	–
310	15	6·0	–	2 × 25	1100	6·5	–
311	15	6·0	–	2 × 25	880	8·0	–
312	15	7·0	–	2 × 10	1400	7·0	–
313	15	6·0	–	2 × 10	1100	7·0	–
314	15	5·5	–	2 × 37	1200	6·0	–
(*c*) *Treatment with boiled pregnant mares' serum*							
315	15	5·5	–	2 × 25	340	6·0	–
317	15	6·0	–	2 × 25	380	8·0	–
318	15	5·5	–	2 × 10	380	7·0	–
319	15	4·5	–	2 × 18	360	6·0	–

* – Sudanophobe zone present, lipoids absent.
\+ Lipoids present in cortex and disappearance of sudanophobe zone.

The experiment with the boiled serum shows clearly that the gonadotrophic activity is destroyed leaving the corticotrophic activity unaffected. The histological appearance of control and treated adrenals is shown in Plate I, figs. 1–4.

Table II. *Effect of treating 9-day-old chicks with purified gonadotrophin from pregnant mares' serum*

No. of chicks	Treatment	Average weight in mg. of: Comb	Testes	Thyroid	Adrenals
6	Controls	18·5	7·3	5·0	13·5
5	2 I.U. daily for 7 days	55·6	14·0	5·1	14·0
6	4 I.U. daily for 7 days	94·8	18·6	4·2	16·8
6	16 I.U. daily for 7 days	127·4	55·9	6·3	17·9
8	32 I.U. daily for 7 days	237·7	79·0	5·8	20·3

Table III. *Effect of oestrone treatment on hypertrophy of the remaining adrenal in unilaterally adrenalectomized rats*

Animal no.	Body weight g.	Weight of adrenals: Right mg.	Left mg.	Days between removal of right and investigation of left adrenal	Compensatory hypertrophy % wt.
(*a*) *Untreated controls*					
57	163	12	16	13	33
59	175	13	22	13	69
60	175	12	19	16	58
61	220	11	16	16	45
63	212	10	16	12	60
64	189	14	23	12	64
65	180	14	19	12	50
66	210	12	18	12	50
(*b*) *Treated with 50µg. oestrone daily*					
49	194	10	24	16	140
50	212	10	25	16	150
51	180	10	31	16	210
52	228	10	35	16	250
53	195	12	19	15	58
54	223	11	25	13	127
55	173	11	19	15	73
56	228	14	23	12	64
(c) *Treated with 1·2 mg. oestrone daily*					
25	170	13	28	15	115
26	220	12	23	15	92
27	185	14	26	10	86
28	204	11	42	12	290
29	187	11	25	15	127
30	204	12	34	15	183
31	170	10	35	14	250
32	226	13	32	15	146

Table II shows the increase in adrenal weight produced in 9-day-old chicks by injections of a purified pregnant mares' serum preparation; in this case there is a more definite relation between dose and response.

These results clearly indicate that there are two separate corticotrophic factors present in pituitary extracts and it has been found possible to demonstrate their independent existence by using oestrone injections. The second adrenal of a unilaterally adrenalectomized rat hypertrophies, increasing in weight by about 70 %. If oestrone is injected after removal of the one adrenal the hypertrophy of the remaining gland is much more pronounced and its weight may increase by 250 %. Oestrone, however, when injected in unilaterally adrenalectomized hypophysectomized rats causes no adrenal hypertrophy so that the effect in intact rats must be due to stimulation of the pituitary to produce corticotrophin. In the animals in which oestrone does increase the adrenal hypertrophy there is no evidence of increased secretion of the factor responsible for disappearance of the sudanophobe zone; in fact sudanophil lipoids may disappear completely from the cortex during the oestrone treatment. It thus appears that oestrone stimulates the pituitary to secrete the factor increasing adrenal weight but does not affect the factor causing disappearance of the sudanophobe zone in hypophysectomized rats (see Table III).

DISCUSSION

These experiments strongly suggest the presence of two different factors in pituitary corticotrophin—one affecting adrenal weight, the other the distribution of lipoid in the cortex. The former alone is present in pregnant mares' serum and it is quite distinct from the gonadotrophin present in the serum.

The absence of correlation between dose and response of the factor increasing adrenal weight is probably explained by the differing degrees of adrenal atrophy present in different rats. The adrenal weight falls from 12 to 6–8 mg. in the first 10 days after hypophysectomy and in the next 3 weeks falls still further to 3–5 mg. It is probable that the response of the adrenal is dependent on the initial weight of the gland. This would explain the fact that the dose is related to response in the 9-day-old chick.

The physiological function of the two factors cannot yet be suggested though their distinction is interesting in view of the facts recently established that the adrenal cortex produces more than one hormone.

SUMMARY

The weight of the adrenal gland in hypophysectomized rats and in intact 9-day-old chicks is increased by injections of pregnant mares' serum. This activity of the serum, unlike the gonadotrophic activity, is not destroyed by boiling.



Fig. 2

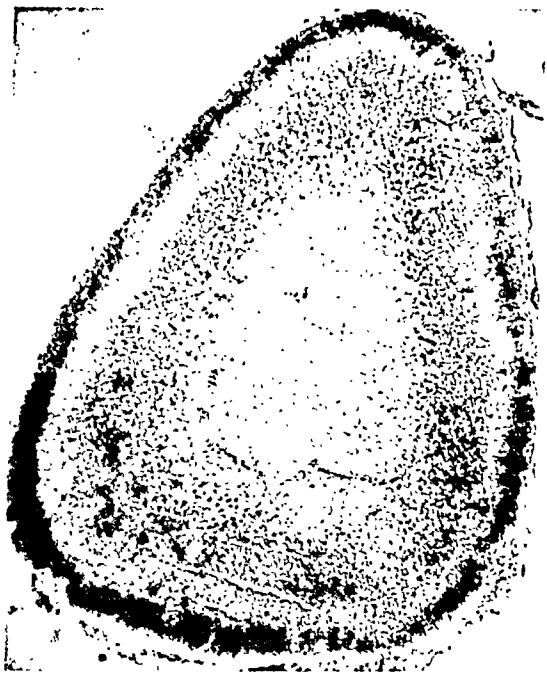




Fig. 1



The substance responsible, unlike the corticotrophin present in anterior pituitary extracts, does not restore the lipoid content of the adrenal cortex in hypophysectomized rats.

It is concluded that there are two different components of the corticotrophin in the pituitary gland and it is shown that oestrone injection stimulates secretion by the pituitary of the adrenal-weight-increasing factor without stimulating secretion of the factor affecting the cortical lipoids.

We are very grateful to Messrs Organon Ltd., London, for completing the organic preparations used in this investigation.

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Description of Plate I

Figs. 1, 2. Adrenals from the same rat. The left adrenal (fig. 1) was removed 10 days after hypophysectomy. The right adrenal (fig. 2) was removed 7 days later after daily treatment with pituitary corticotrophin.

Figs. 3, 4. Adrenals from the same rat. The left adrenal (fig. 3) was removed 10 days after hypophysectomy. The right adrenal (fig. 4) was removed 7 days later after daily treatment with an extract of pregnant mares' serum.

Frozen sections stained with haematoxylin and eosin. Magnification (× 25) is the same in all cases.

FURTHER OBSERVATIONS ON THE ROLE OF PROGESTERONE (PREGNANEDIOL) AND OESTROGEN IN PREGNANCY

By A. M. HAIN

From the Institute of Animal Genetics, Edinburgh University

(*Received 19 July 1941*)

In the previous report [Hain, 1940], with a view to examining hormone changes associated with the birth mechanism, the author investigated the output of pregnanediol and of the two combined oestrogens in women approaching parturition and also in one woman throughout the whole of a normal pregnancy. The larger series comprised both normal and toxaemic patients, none of whom received therapy; in no instance was the amount of free oestrogen excreted estimated.

The present series investigates the problem of pregnancy and parturition from a different angle, in that it seeks to ascertain the hormone output associated with abortion. The series consists of women with histories of previous miscarriage or showing signs of threatened abortion in the pregnancy investigated, and falls into two parts: the first consisting of five women in whom both the oestrogen and the pregnanediol outputs were ascertained at very frequent intervals, and the second of over 100 patients in whom, partly owing to the difficulty of doing biological assays under war conditions, only the pregnanediol output was investigated at intervals of 3–4 weeks throughout pregnancy. In some instances it was possible to correlate the pregnanediol output with Aschheim-Zondek tests in the same individual. In a limited number of cases hormone analyses were made for specific reasons, e.g. prolonged sterility, diabetes, pregnancy toxaemia.

METHODS

Extraction of oestrogens from urine. The method of hydrolysis and separation of the combined oestrogens used in the two previous series [Hain, 1939, 1940] and described in 1939 was again employed. As the hormone concentration is low during the early months of pregnancy, a 48-hr. output of urine was always used. Of this approx. 800 ml. were used for combined oestrone, 400 ml. for combined oestriol and 1000 ml. for pregnanediol. Towards the end of pregnancy, when it was necessary to estimate also the amount of free oestrogen, 500 ml. of the urine previously allocated to pregnanediol were used, since, owing to the increased excretion

of pregnanediol, less urine was required for this latter analysis. The method of separation of free oestrogen was the same as that for combined, but without hydrolysis. An aliquot portion of urine was acidified with concentrated hydrochloric acid (generally 40 ml. to a litre of urine) and was then extracted with crystallizable benzene in a continuous extraction apparatus for 24 hr. Its subsequent preparation and assay on ovariectomized mice were on the lines employed for the combined oestrogens in the present and previous series [1940]; the activity was expressed in terms of the international standard of oestrone as determined by biological assay [Hain & Robson, 1938].

Pregnanediol. The gravimetric method for the determination of sodium pregnanediol glucuronide in urine described by Venning [1937, 1938] and used by the author on the previous series was again employed. All values are given in terms of pregnanediol by means of the formula prescribed by Venning, and, as in the case of the oestrogens, the output is estimated per 24 hr. Venning's range of excretion in eight cases of normal pregnancy [1938] was adopted as the standard of comparison for pregnanediol values.

The clinical history of cases is given as fully as the circumstances allow.

FIRST SERIES

This small group of five consists of: two sisters who had had two and three miscarriages respectively and who carried through successfully the pregnancies investigated; one woman with a similar past who aborted at 22 weeks; a fourth woman with only one previous miscarriage who was followed throughout a successful pregnancy; and a normal woman in whom daily analyses were made during the last 12 days of pregnancy. This patient served as a control as regards the output of free oestrogen which might have been of an abnormal nature in the women who had previously miscarried. All the members of this group, except the normal control examined during the last 12 days of pregnancy, received therapy on account of their miscarriages.

The author wishes to record her indebtedness to the two sisters, P 7 and P 8, for their devoted perseverance in the troublesome task of urine collection over a period of 9 calendar months. The history of P 7 is as follows:

Case P 7

History. At time of birth of child, aged 36: two miscarriages in 2 years: at 6 weeks (1937) and at 6 months (1938), when a live male child was born but died after a few hours. Menstrual cycle 3–4 days every 24–25 days; some dysmenorrhoea but not after marriage; loss considerable.

Therapy. During first two pregnancies thyroid only. During the pregnancy under investigation: (*a*) Desiccated thyroid 2 grains daily except during last month of pregnancy when 3 grains daily, as slight oedema of ankles developed. (*b*) Vitamin E: 300 capsules Fertilol during first 6 months of pregnancy: three

capsules a day for first 3 months, one a day afterwards. (*c*) Progesterone: three injections of 10 mg. each weekly for 2 weeks when 30 days pregnant because of slight threatening of miscarriage. Thereafter 5 mg. weekly till 8th month when 10 mg. weekly for 3 weeks. During last 5 weeks of gestation only one dose of 10 mg. was given, namely, at 3 weeks before parturition when a drop in pregnanediol output caused fears of premature delivery. With that exception, both in P7 and in P8 progesterone therapy was suspended during the last 5–6 weeks of gestation lest its administration should delay parturition. (*d*) Rest: in bed for 3 weeks when discharge at 30 days caused fears of miscarriage; thereafter in bed for one week each month, and for first 5 months lived what she called a 'vegetable existence'.

A healthy, living child weighing 6½ lb. was born on 4 Dec. 1939, 276 days after the last menstrual period, following a labour lasting only 3 hr.; the action of the uterus was 'tremendously strong'.

Experimental procedure and results

Pregnanediol estimations were commenced 10 days after the last menstrual period and before pregnancy could be diagnosed. By the 23rd day of the cycle it could be seen that pregnancy existed, as the total output of pregnanediol was double the output in the luteal phase of a normal menstrual cycle. The details were published [Hain & Robertson, 1939] and demonstrated that it was possible to diagnose pregnancy before a period had been missed and before the Aschheim-Zondek test gave a definite positive reaction.

Estimations of pregnanediol output were continued at intervals of 2–3 days until the 130th day, as some anxiety was felt at the absence of any appreciable rise in output. MacGregor & Stewart [1939] had reported a marked rise in the excretion of pregnanediol between the 60th and 90th days, although no such peak occurred in the chart given by Venning [1938] comprising data of eight normal pregnancies. It is probable that the feature noted by MacGregor & Stewart was abnormal as, in a series of cases investigated, the author has found its occurrence to be associated with anomalous features. From the 130th day until the last month analyses were made once weekly; during the last month of gestation more frequent estimations were made at 3–4 day intervals, and daily during the last fortnight.

Reference to Fig. 1 shows that the pregnanediol output was low throughout the whole of gestation. During the first 4½ months it remained almost stationary, between 10 and 14 mg., whereas, according to Venning, it should have been doubled. The low output is most marked, however, from this stage onwards when, with hardly an exception, the highest point reached is below Venning's [1938] minimum for the same stage. No peak occurred during the 8th and 9th months such as is normally found at this stage and the maximum was about 40 mg. It is interesting to observe,

however, that the rhythmic rise and fall in pregnanediol output which preceded parturition in the author's case 48 [Hain, 1940] was observed here also. A shorter period was covered by this phenomenon, as events appear to have been precipitate during the last 6 days of gestation, the drop in pregnanediol being accompanied by a sensational drop in combined oestrone.

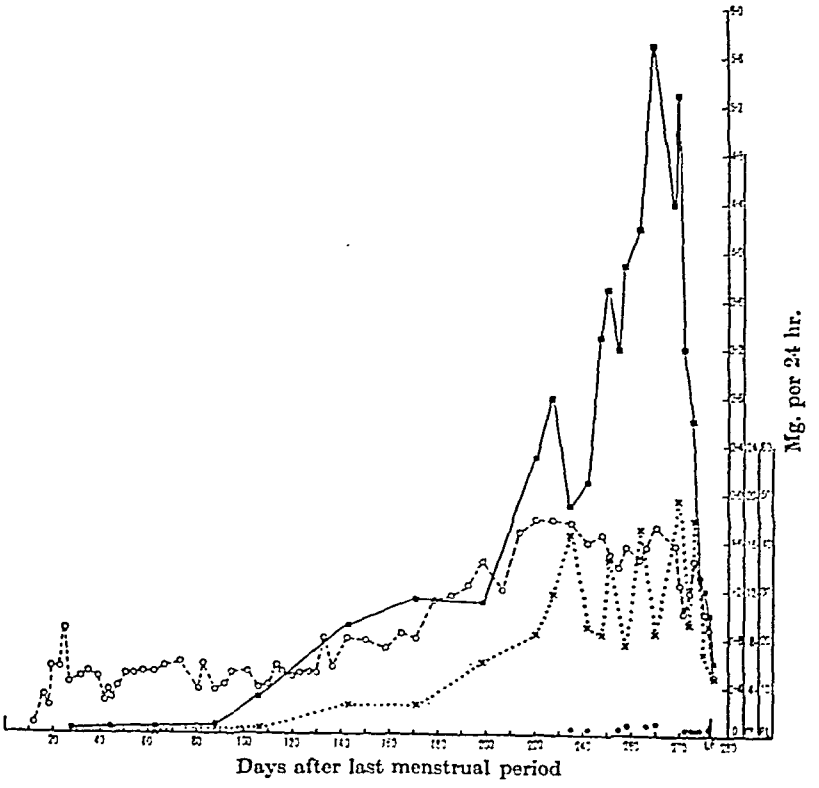


Fig. 1. Case P 7: the excretion of pregnanediol and oestrogens throughout pregnancy. (From the 260th to 280th days double scale is used for greater clearness.) O---O pregnanediol (*PL*); ×·····× combined oestriol (*OL*); ■——■ combined oestrone (*O*); ● free oestrogen (to be read against scale for oestrone). *L* = labour; *P* = parturition.

The combined oestrogen excretion did not present any abnormal features. During the first 3 months the output of both combined oestrone and combined oestriol is low—a normal feature. At the 4th month this begins to rise until, by steps, the output of combined oestrone reaches a maximum of 5·7 mg./24 hr. 11 days before parturition. After an initial fall and rise (indicative of the rhythm previously commented upon), a rather precipitate drop occurs in the subsequent 6 days until only 1·0 mg. is excreted on the day that labour commences. The curve for oestriol is

less steep, although fairly high values are reached, its nature being determined probably by the pregnanediol excretion.

At intervals from the 235th day onwards the amount of free oestrogen excreted was also determined. During the last 6 days of gestation daily estimations were made (with a single exception owing to a small urinary output), as it is during this time that, according to Cohen, Marrian & Watson [1935], there occurs a rise in the free forms of oestrogen which, they suggest, may prove to be an important factor in the initiation of labour. However, the amount of free oestrogen recovered remained remarkably stationary, as can be seen on reference to Fig. 1. It is unlikely that it played any part in the birth mechanism in this patient.

If the data be examined for any light that may be thrown on the possible cause of the previous miscarriages, two features are remarkable: (*a*) *The low pregnanediol output*, indicating deficient secretion of progesterone or a faulty formation of the glucuronide. At the time when the placenta should, presumably, be taking over the secretory function of the ovaries—namely, at $3\frac{1}{2}$–4 months—the previous level of excretion was barely maintained; subsequent levels caused anxiety throughout the whole of pregnancy and rendered the outcome in doubt up to the end. The only encouraging feature was the normality shown by the combined oestrone output. (*b*) *Combined oestrone output.* This, however, reached such levels as to suggest that a sudden marked rise in oestrogen with an equally sudden fall might have played as great a part in previous miscarriages as a deficient progesterone metabolism. The shortness of the labour process can hardly be ascribed to the high oestrone level attained, in view of the absence of any such correlation in the cases previously analysed [Hain, 1940] but may denote a highly sensitive uterus—another contributory factor to abortion.

Case P8

History. At time of birth of child, aged 30; three miscarriages in 2 years: (1) Feb. 1937 at $2\frac{1}{2}$ months; (2) Nov. 1937 at 6 weeks; (3) Dec. 1938 at 6 weeks. Appendicectomy Feb. 1938. Patient curetted after each miscarriage. No treatment given during first pregnancy but one capsule of vitamin E was taken daily between first and second pregnancy. During third gestation four vitamin E capsules were taken daily and two injections of 5 mg. progesterone were given before miscarriage occurred. Patient did not rest in bed.

Therapy during this (4th) pregnancy: (*a*) Progesterone: 10 mg. weekly from end of 2nd month to $7\frac{1}{2}$ months when reduced to 5 mg. weekly for 5 weeks. No progesterone given after 35 weeks. The patient lay in bed from 14 May to 25 Sept. 1939 and took things very quietly when she got up. No vitamin E capsules and no special diet, but calcium, iron and vitamin A were administered. Owing to a rise in blood pressure to 140/100 and trace of albumen in the urine 1 week before term, patient was put on light diet until delivery. Delivery was quick and easy and took place on 23 Jan. 1940.

Experimental procedure

Pregnanediol estimations carried out during the whole of the luteal phase of the menstrual cycle preceding conception showed that this patient possessed normal ovarian function. From the 41st day after this menstrual period the pregnanediol output was estimated at weekly intervals until the 260th day, when estimations were made every 4 days and then daily until term. The combined oestrogens were determined monthly for 7½ months from which time weekly determinations were made until the last fortnight when daily values were obtained. Values for free oestrogen were ascertained at various times from 180 days onwards and almost daily during the last week of pregnancy. In addition, the amount of gonadotrophic hormone excreted at the end of gestation was determined by Frank's modified method [1939], immature female rats being used for the assay.

Results

The data are charted in Fig. 2. Throughout the whole of pregnancy P 8's pregnanediol output was higher than that of P7, and at no time was anxiety felt regarding her progesterone secretion. From approximately 6 months the values step up gradually and rhythmically until a peak of 100 mg./24 hr. is reached on the 262nd day, from which time the output rises and falls rhythmically until labour occurs at a 34 mg. level. The definitely rhythmic character of both the pregnanediol and combined oestrone output is clearly shown in Fig. 2*a*.

The oestrogens present interesting features. The extraordinary rise in oestrogen excretion between the 211th and 224th days culminating in the peak of the 217th day occurred in all three forms: combined oestrone and oestriol and free oestrogen. It has already been pointed out [Hain, 1940] that parturition is preceded by a marked rise in the amount of oestrogen excreted in combined form, this rise occurring usually 12–18 days before parturition, to be followed by rhythmically decreasing values. The features presented by the oestrogens between the 211th and 240th days were so suggestive of impending parturition (or abortion) that the patient was asked if any unusual circumstances existed at the time which might have been responsible. It transpired that during the week before the peak excretion of the 217th day her husband left with the British Expeditionary Force for France and that a friend staying in her house aborted after a few days' illness. These two circumstances affected the patient seriously and she was nervous lest she too should abort. It is of interest that in the same patient lactation ceased 3 months after labour coincident with another emotional upset. It seems likely that in those instances in which abortion occurs as the result of severe mental disturbance, the effect is produced by emotional influence passing from the hypothalmus to the

pituitary and thus affecting the secretion of oestrogen. I am indebted to Prof. Johnstone for this suggested interpretation, which seems justified by the facts of this case.[1]

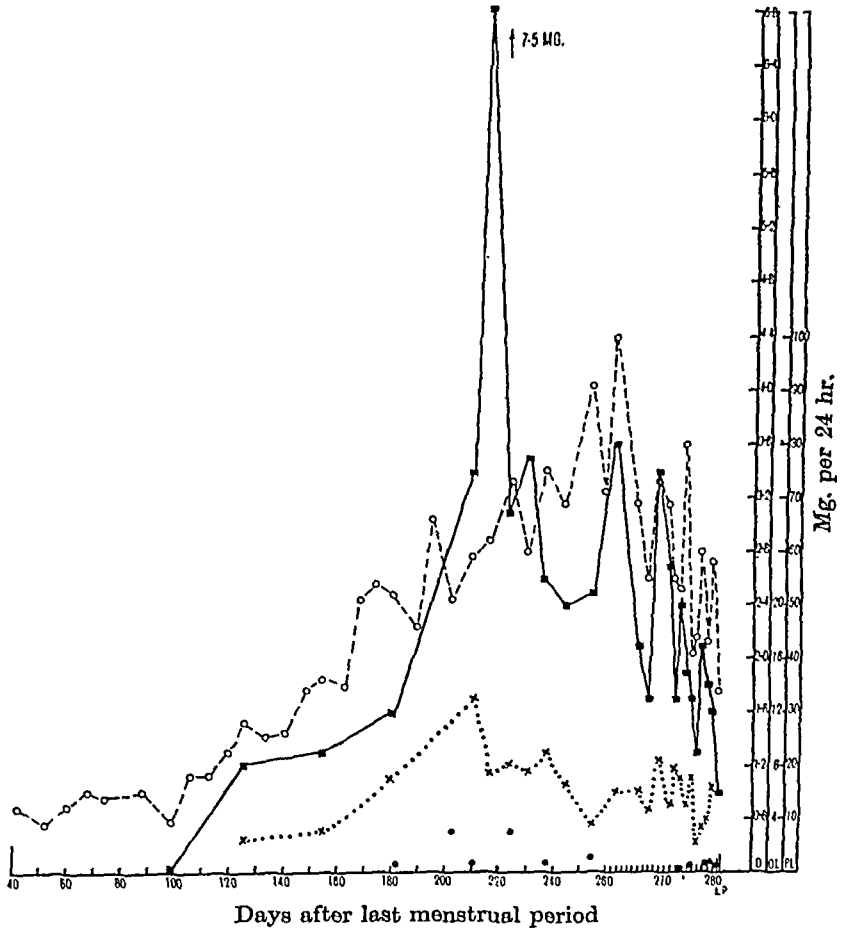


FIG. 2. Case P 8: the excretion of pregnanediol and oestrogens throughout pregnancy. (From the 260th to 280th days double scale is used for greater clearness.) O – – – O pregnanediol (*PL*); × ····· × combined oestriol (*OL*); ■——■ combined oestrone (*O*); ● free oestrogen (to be read against scale for oestrone). *L* = labour; *P* = parturition.

Reference has already been made to the observation that Cohen *et al.* [1935] found a rise in the free forms of oestrogen at the approach of labour. A similar finding in a case of abortion was recorded by Palmer [1938]. The absence of any increase in free oestrogen in P 7 and in case 70 (below), and its existence in P 8 at a time when combined oestrone was excreted in abnormally large amounts, suggest that this may be one of the factors responsible for abortion in certain cases and for parturition in others, but

[1] I am informed by the Edinburgh Pregnancy Diagnosis Laboratory that when profound emotional disturbance exists in a non-pregnant woman, the Aschheim-Zondek test sometimes gives a weak positive reaction, indicating an exceptionally high pituitary activity.

it is clear that it is not a necessary participant in the process. In P 8 the excretion of free oestrogen remained almost stationary at a low level during the 6 days preceding labour. Regarding the remaining oestrogen—combined oestriol—it will be noted on reference to Figs. 2 and 2*a* that its outline and that of combined oestrone are similar, from which it would appear that the amount of oestriol excreted is determined by the amount of oestrone available for conversion by progesterone rather than by the amount of progesterone secreted.

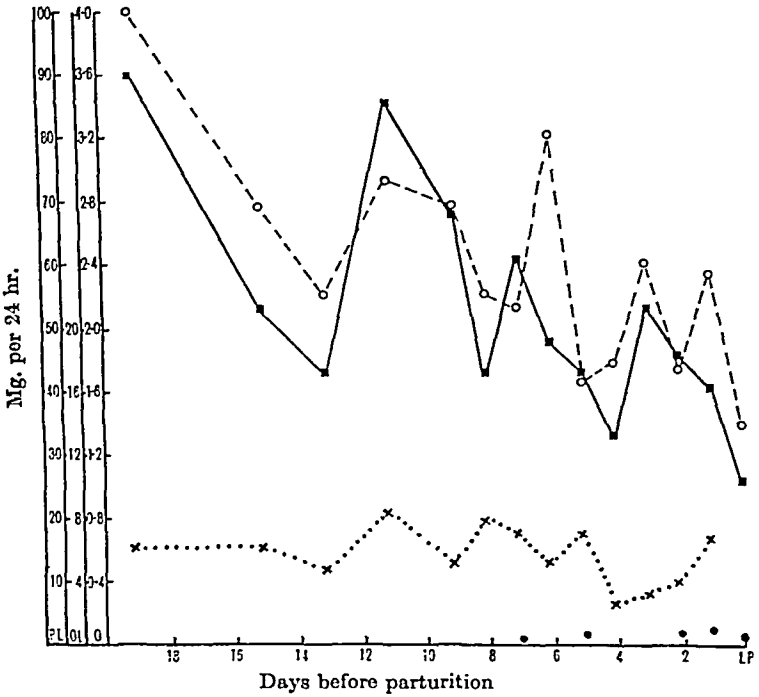


FIG. 2*a*. Case P 8: the excretion of pregnanediol and oestrogens during last 19 days of pregnancy, showing pre-parturitional rhythmic swing. For key to symbols see Figs. 1 and 2.

In view of the obvious rhythm present in the rise and fall in hormone elimination in the three combined forms during the last few days of pregnancy, a rhythm which has been observed as an integral part of the pre-labour process (Hain [1940] and in four cases described and illustrated here), it appeared desirable to ascertain to what extent this phenomenon could be attributed to waning pituitary activity. Such a theory was recently put forward by Robson [1940*a*] as a possible explanation of the mechanism of parturition in the rabbit, and the rhythm that has been here described certainly points to some such control. The equivalent of 40 ml. urine taken from the 24 hr. specimens passed 8 days and 48 hr before

labour, in the case of P 8, gave almost identical results when injected into immature female rats. There was thus no indication of a marked drop in gonadotrophic hormone excretion during the last 8 days of pregnancy in association with the drop in combined oestrogen and pregnanediol. It is to be hoped that, with the arrival of accurate analyses of hormone concentration in the blood, this problem will be clarified.

The three miscarriages recorded by this patient occurred before the 3rd month, at a time when progesterone secretion is naturally low and when pregnancy can be most readily interrupted. The data suggest that, although a progesterone deficiency might have been responsible for these mishaps, the susceptibility of the patient's pituitary to emotional influence might have contributed in considerable measure to the issue as it so nearly did in the pregnancy under review. As having bearing on the familial character of hormone deficiency it is of interest to note that a third sister had a similar history of miscarriages before success was attained; also the mother of P 11, the next case to be described, had five miscarriages before she gave birth to live children.

Before leaving these two cases, of which such full data are available, it may be pointed out that in neither of the patients was there any fluctuation in hormone output observable at what might correspond to monthly periods. It might be argued that any possible fluctuation was obscured owing to additional therapy and rest being taken at these times. However, when one considers the extent to which the length of the menstrual period varies in the same individual, it is unlikely that after 3 months at most there was any coincidence of date and treatment. A cyclic excretion of oestrogen during pregnancy, with peaks every 24–29 days, has been reported by Browne & Venning [1936], Smith & Smith [1936], and of pregnanediol by Bachman, Leekley & Hirschmann [1940]. The evidence is not clear, however, and in the single case shown separately by the last-named authors the peaks of pregnanediol were at intervals of 2, 3, and 4 weeks respectively.

Case P 11

In the two patients already described, pregnancy had a successful termination; in P 11 a miscarriage occurred in spite of similar therapy, which, however, was not commenced until the 3rd month, making the cases not strictly comparable. The data are as follows:

History. Aged 28 in Sept. 1939; three miscarriages in 3 years: (1) at 6½ months (1935), (2) at 2 months (1936), (3) at 5½ months (1937)—in the two latter years, after extra fatigue.

Therapy. Iron, calcium, thyroid, vitamin E and progesterone during 3rd month, before coming to Scotland. From 4th month onwards vitamin E capsules three daily, 2 mg. progesterone every 3rd day, increased to 5 mg. × 2 during

he week corresponding to suppressed periods when the patient stayed in bed; lso thyroid and calcium.

Outcome. Miscarriage occurred at 22 weeks in spite of administration of 35 nits of progesterone and morphia during the 24 hr. following the first appearance of the symptoms. Prof. Johnstone, to whom I am indebted for the case, was of the opinion that there was a sudden increase in the liquor amnii which was certainly excessive; hydramnios was present and there might have been some ovular defect, although the foetus appeared normal. This was the patient's fourth miscarriage; a fifth occurred in 1940.

Experimental procedure

Hormone estimations were begun at 3½ months and terminated with the miscarriage at 5½ months. Pregnanediol analyses were made at weekly intervals and the combined oestrogens monthly. Three days before pregnancy terminated an estimation was made of the free oestrogen excreted.

Results

The data in Fig. 3 show that P11's excretion of pregnanediol was such that there was no reason to be anxious about her condition, or to anticipate the disastrous outcome which came so suddenly. Indeed, 3 days before her miscarriage the pregnanediol output (at 34 mg. in 24 hr.) was entirely satisfactory as can be seen when comparison is made with other records at the same stage; for example, P7 = 19·5 mg., P8 = 36 mg., and Venning [1938], 22–43 mg. (range of normality for 5–5½ months' pregnancy). Similarly, the combined oestrone was not unduly high, the figure of 1·4 mg. a fortnight before abortion comparing with 0·9 mg. in P7 and 1·3 mg. in P8 at the same stage of pregnancy. Nor did a rise in free oestrogen occur such as Palmer [1938] observed in his case: this was 0·003 mg. 3 days before abortion.

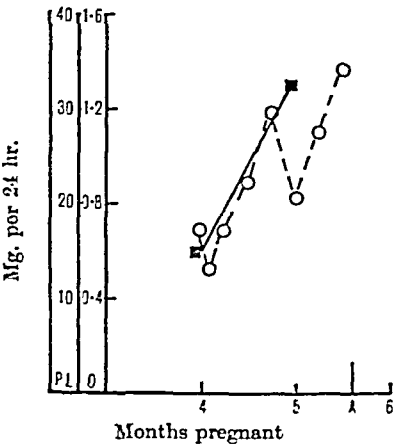


FIG. 3. Case P11: the excretion of pregnanediol and combined oestrone. Abortion (*A*) at 22 weeks while receiving therapy. Note rhythm in pregnanediol output. O - - - O pregnanediol (*PL*); ■——■ combined oestrone (*O*).

There is, however, one obvious feature illustrated in Fig. 3, that is, the rhythmic nature of the pregnanediol output, a feature absent from the other charts at the same stage and one which, the author suggests, denotes a control that has frequently been observed before the onset of labour. Later on in this report several other instances will be given of miscarriage occurring at high levels of pregnanediol excretion. Only frequent analyses could determine whether such a control, as was present in the case of P11,

was responsible for the outcome in these cases also. It is readily understood that when such an abnormality is coupled with a possible ovular defect, even large amounts of progesterone would probably be unsuccessful in maintaining pregnancy.

At the time of this patient's fifth miscarriage 4 months later, her output of pregnanediol (12 mg. at almost 2½ months) was again satisfactory for the stage of pregnancy. The oestrogen excretion was not ascertained, but it is interesting to note that the Aschheim-Zondek test 3 days before the miscarriage (and 2 days before the pregnanediol analysis) was strongly positive, suggesting normal activity on the part of the placenta as regards chorionic gonadotrophic hormone. There was, thus, no fall in excretion of the latter such as sometimes occurs as a prelude to abortion.

Case 70: Normal control

In view of the possibility that the absence of any rise in the excretion of the free form of oestrogen at the approach of labour was a feature common only to the abnormal cases above described, the hormone output of a normal woman, receiving no therapy, was investigated during the last 12 days of pregnancy. Fig. 4 shows that the output of free oestrogen remained stationary at a very low figure and cannot have played any part in the birth mechanism. Other noteworthy features, held in common by the cases already described here and in 1940 [Hain, 1940], are: the similarity in outline of the curves of the combined oestrogens and of pregnanediol, their rhythmic rise and fall, and their falling trend during the days immediately preceding labour. As has been noted in other cases elsewhere [Hain, 1940] the labour specimen of urine contained more combined oestrone than was excreted 2 days earlier.

Case P40

Clinical details. Miscarriage at 6½ months, 1939, due to intra-uterine death, cause unknown. Present pregnancy: slight losses of blood at 1, 2 and 2½ months. Progesterone: 5 mg. twice weekly from beginning of 3rd month to end of 7th; also three vitamin E capsules thrice daily. Live child 27 Oct. 1940, at 8½ months.

Of the oestrogens, only combined oestrone was estimated in this patient, and unfortunately, owing to difficulties in submitting a second day's specimen of urine in the latter part of pregnancy, the data for oestrone are scanty at that stage.

According to the dates given by the patient, she was 2½ months pregnant at the time of the first hormone estimations, but when labour was induced at what would have been 9½ months, the nature of her pregnanediol curve showed that labour was not yet due, and this was confirmed by the child being slightly premature at birth (almost 5 lb. in weight and 16 in. long). As an error of approximately 1 month was made, the chart (Fig. 5) has been brought into line with later information.

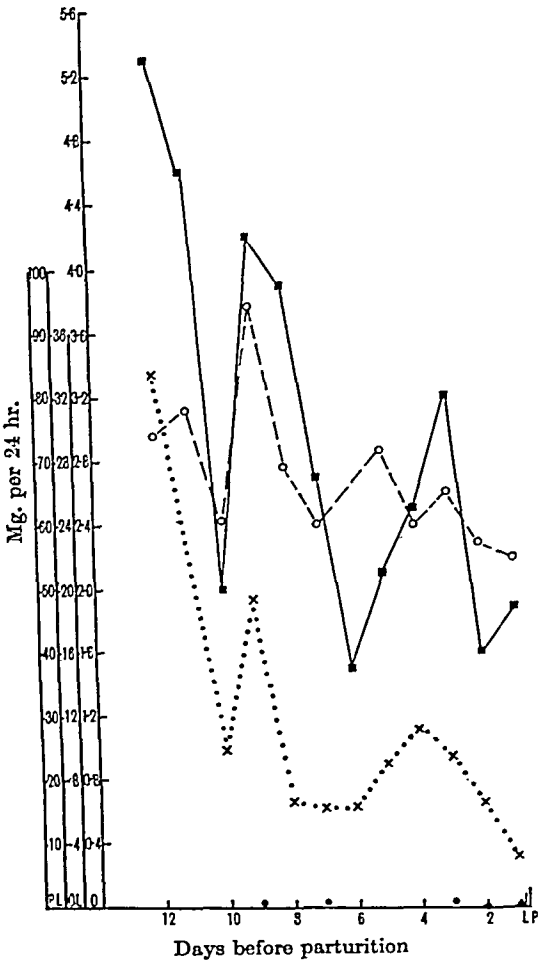


FIG. 4. Case 70: the excretion of pregnanediol and oestrogens by a normal woman during last 12 days of pregnancy. O---O pregnanediol (*PL*); ×·····× combined oestriol (*OL*); ■——■ combined oestrone; ● free oestrogen (to be read against scale for oestrone). *L*=labour; *P*=parturition. Note pre-parturitional swing.

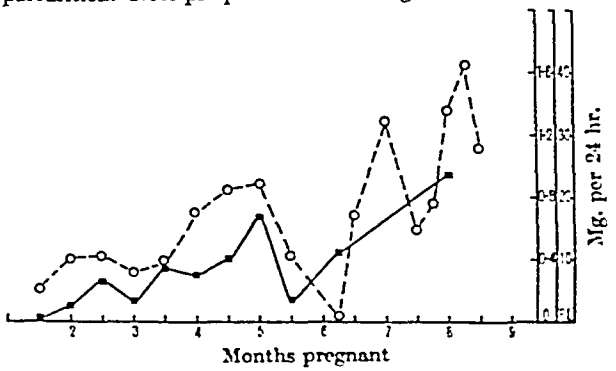


FIG. 5. Case P40: the excretion of pregnanediol (*PL*) and combined oestrone (*O*) throughout pregnancy. For key to symbols see Figs. 1, 2 and 4.

The outstanding feature about P40 (Fig. 5) is the extraordinary drop in pregnanediol output at $5\frac{1}{2}$–$6\frac{1}{4}$ months, only a trace being found on the latter date. Any loss in potency as the result of 2–3 days in transit (the patient had gone to the south of England for the time being) would be infinitesimal and could not have been responsible; it will be observed that the combined oestrone shared in the drop at $6\frac{1}{4}$ months. A similar, though much less marked, fall in pregnanediol output occurred in case P62 to which reference may be made (p. 39); in both instances pregnancy was uninterrupted. Cope [1940*a*] cites a similar case in which the pregnanediol output fell to nil at 19 and 21 weeks without interfering with pregnancy, although symptoms of threatened abortion were present. The low pregnanediol excretion throughout pregnancy suggests that the patient's previous miscarriage was due to a deficient progesterone secretion.

SECOND SERIES

The second part of this study deals with the following:

(1) *Symptoms of threatened abortion:* the gonadotrophic hormone and pregnanediol output associated are examined singly and together; Aschheim-Zondek tests related to the outcome of pregnancy.

(2) *Abortion:* cases of abortion at high and at low levels of pregnanediol output.

(3) *Pregnanediol values throughout pregnancy* as a guide to therapy and in relation to normal development, function and prognosis: (*a*) medium normals, (*b*) medium high normals, (*c*) low normals, (*d*) drops in early pregnancy.

(4) *Pregnanediol values* in (*a*) toxaemia, (*b*) diabetes, (*c*) foetal death.

(5) *Therapy*—progesterone, vitamin E, rest.

(6) *Sterility.*

(7) *Interesting cases.*

Threatened abortion

Comparison of Aschheim-Zondek tests with pregnanediol output

As a general rule, a positive Aschheim-Zondek finding in early pregnancy is held to denote a normal pregnant condition, yet of forty-five women for whom positive results were returned, nineteen, or 42 %, aborted: half of these within 20 days of the test and one actually on the day that the positive finding was obtained.

A weakly positive Aschheim-Zondek reaction before the 4th month is generally considered indicative of a possible threatened abortion, yet of thirteen women in whom this finding was obtained only five, or 38·5 %, aborted. Moreover, in three out of the ten women who went to term, the Aschheim-Zondek reaction was very weak. Of four weak positives in patients after the 4th month two aborted and two had live babies.

The failure of a weakly positive Aschheim-Zondek reaction to afford a reliable guide as to the probable outcome of pregnancy is more forcibly brought out in the following: of twenty-four women who aborted, a weak positive at 2–4 months was returned in only 21 %; of thirty-four women who had viable children the same test finding was obtained in 23·5 %.

It was these facts which prompted an investigation of the gonadotrophin and pregnanediol output associated with symptoms of threatened abortion. It is commonly held that the condition is due to a deficiency of pituitary and corpus luteum function. Accordingly, data were examined under both headings to see if the condition could be related to a deficiency of either or both substances. Symptoms of threatened abortion such as bleeding, staining or brown discharge were present both before and at the time of the tests.[1] Of thirty-five such women, in whom both the pregnanediol and gonadotrophin outputs were ascertained, only eighteen had live children (in two others pregnancy is still running); in fifteen abortion occurred, in many cases after an interval of less than 14 days. Thus symptoms of threatened abortion in early pregnancy resulted in abortion in 43 % of cases.

Aschheim-Zondek tests. Of forty Aschheim-Zondek tests made for the thirty-five women, only twelve weak or very weak positive findings were returned, whereas strong or standard positives were obtained in twenty-eight cases or in two-thirds of those examined. It is thus clear that the symptoms of threatened abortion cannot be attributed, even in the majority of patients, to low gonadotrophin secretion.

An analysis of the data given for the fifteen cases that terminated in abortion demonstrates that imminent abortion (i.e. in 1–20 days) was only once associated with a weak positive Aschheim-Zondek finding; and, strangely enough, the associated yield of pregnanediol was abnormally high (38 mg. in 24 hr. in a 3½ months' pregnancy); abortion occurred 13 days later (case P 21). Other records are: a standard positive on the day abortion occurred (P 84) when the foetus was already dead; a weak positive on the day before a premature delivery at 8 months (P 174)—in both these cases the pregnanediol values were again high; a strong positive 8 days before abortion and seven positives in women who aborted 10–20 days later. Of the seven women who aborted 4–8 weeks after the test, five gave strong or standard positive reactions.

On the other hand, among seventeen women who gave birth to live children at term the distribution of twenty Aschheim-Zondek findings at 2–3½ months was: eight weak or very weak positives and twelve strong or standard positives, representing an adverse report in 40 % that terminated successfully.

[1] The author is indebted to the Pregnancy Diagnosis Laboratory for the Aschheim-Zondek findings.

Pregnanediol output. Of the forty pregnanediol analyses made for the same thirty-five women, twenty-six showed the output of pregnanediol to be within the normal range as given by Venning [1938]; in five other instances the output was even higher than Venning's maximum for the stage of pregnancy, and on only nine occasions was it low, of which there were three when only a trace of pregnanediol was recovered. It follows that neither could a deficiency of progesterone account for the symptoms of threatened abortion in the majority of the cases reviewed. Indeed, in 61 % of cases the gonadotrophin, and in almost 80 % the pregnanediol output was of a satisfactory character. The latter figure is somewhat reduced if one examines the data for all cases of threatened abortion in which the pregnanediol output was ascertained, irrespective of whether Aschheim-Zondek tests were made or not. The total of such cases is 64, and, as is shown in Table I in 61 % of these satisfactory pregnanediol outputs were obtained.

Table I. *Pregnanediol values in cases of threatened abortion*

Total no. of cases	Normal values	High values	Low values	Percentage satisfactory	Percentage low
64	31	8	25	61	39

If the pregnanediol figures for the smaller series (of thirty-five women) be examined in relation to the outcome of pregnancy (Table II) it will be seen that, of twenty-three women in whom the pregnanediol output was normal, seven aborted: when the values were higher than normal, four out of six aborted. As abortion was frequently related to values obtained at a later period than the gonadotrophin finding such cases are considered in the list of abortions at high or satisfactory pregnanediol values in Fig. 7, Table IV.

Relation between gonadotrophin and pregnanediol outputs. Since it may be contended that abortive symptoms might be due to an imbalance between the two substances, the relationship between the gonadotrophin and pregnanediol outputs in the same person has been analysed in Table II (A = abortion, L = live child). The stage of pregnancy at which the Aschheim-Zondek tests were made has been inserted in order that the strength of the reaction might be appreciated; it will be remembered that in normal pregnancy this diminishes after the 4th month.

It is clear from the data in Table II that abortion, or the symptoms of abortion, in the cases examined was not accompanied by a low concentration of both substances or confined to a low output of one of them.

An abnormally high pregnanediol output was associated with a low excretion of gonadotrophin in four of the patients, in three of whom abortion occurred in 2, 11 and 16 days after the high pregnanediol output.

Table II. *Aschheim-Zondek findings related to pregnanediol output in the same person, with outcome of pregnancy: thirty-five cases*

Very weak A.Z.+ high pregnanediol			Weak A.Z.+ high pregnanediol			Standard A.Z.+ high pregnanediol		
Months pregnant	Case no.	Result	Months pregnant	Case no.	Result	Months pregnant	Case no.	Result
3	P43	A	3½	P16	A	2	P44	L
			3½	P21	A	4¼	P84	A
			3½	P37	L			

Standard A.Z.+ normal pregnanediol			Strong A.Z.+ normal pregnanediol			Weak A.Z.+ normal pregnanediol		
Months pregnant	Case no.	Result	Months pregnant	Case no.	Result	Months pregnant	Case no.	Result
2¾	P27	L	4	P19	A	2½	P41	L
3	P28	L	3	P84	A	3	P41	L
1¼	P50	A	3	P14	L	2½	P98	L
3	P50	A	2	P34	L	3¼	P134	L
2¾	P52	L	3	P124	L	5	P30	L
5	P53	A	2½	P138	*	3½	P120	L
3¼	P56	L				8	P174	A
2¾	P58	A						
2	P62	L						
2½	P20	A						
3¼	P123	L						
7¼	P133	L						

Very weak A.Z.+ normal pregnanediol			Standard A.Z.+ low pregnanediol			Strong A.Z.+ low pregnanediol		
Months pregnant	Case no.	Result	Months pregnant	Case no.	Result	Months pregnant	Case no.	Result
4	P41	L	2	P40	L	2½	P141	A
			5	P65	A	3	P141	A
			2½	P118	A	2½	P151	A
			2	P23	A	1¼	P152	*

* Pregnancy still running.

In the groups in which one would expect few, if any, abortions, that is, in those in which the Aschheim-Zondek test was a strong or standard positive and the pregnanediol output normal to high, six out of the eighteen patients aborted. Among these six, abortion occurred 1, 4, 7, 10 and 10 days after a highly satisfactory output of pregnanediol, in one on the day of a positive Aschheim-Zondek finding, and in four others after intervals of 3, 12, 13 and 16 days. The sixth woman (P50) did not abort until 22 weeks, on a day on which the output of pregnanediol was entirely satisfactory (38·5 mg.).

A weak or very weak positive Aschheim-Zondek test plus a normal pregnanediol output at 2½–5 months resulted in live children in all of the five

cases recorded. P174, who was already in labour when the Aschheim-Zondek test and pregnanediol analyses were made, had had five abortions at 4–8 months, and on this occasion foetal death at 8 months was associated with a funic presentation and abnormally long cord (32 in.) containing a true knot. During labour, which was spontaneous and painless, the pregnanediol output was 78 mg./24 hr., and the Aschheim-Zondek test weakly positive; the patient delivered herself the following day.

In only five patients was abortion associated with a low pregnanediol excretion; in three of these the Aschheim-Zondek reaction on the same specimen of urine was strong or standard positive, in the remaining two cases the test was made on an earlier specimen but was also positive. Abortion occurred 13–20 days after the low pregnanediol output. It was observed in a number of cases of imminent abortion that the pregnanediol output fell before the Aschheim-Zondek finding became negative or even weakly positive. In some instances of threatened abortion in early pregnancy marked fluctuations in pregnanediol output were observed, but this was not a regular feature.

Effect of therapy. Therapy will be dealt with briefly as it is discussed more fully at a later stage. From Table III it is seen that of thirty-five women receiving various kinds of therapy for symptoms of threatened

Table III. *The outcome in thirty-five cases of threatened abortion with or without therapy*

Vitamin E + rest	Vitamin E + progesterone	Vitamin E + progesterone + rest	Progesterone + rest	Rest only	No therapy
4L 2A	1L	4L 4A	3A	1L (not viable)	5L 11A

L = Live; A = Abortion

abortion and observed until parturition or abortion occurred, fifteen had live children and twenty aborted; of the sixteen who did not receive any therapy five had live children and eleven aborted, a percentage ratio of abortions in the two groups of 57:70. Regarding the untreated group, it should be noted that such patients generally came into the doctor's hands too late for treatment. It is of interest, however, that in only four of the eleven patients aborting without therapy were the pregnanediol values low; in contrast, one woman, without therapy, who excreted very low values throughout pregnancy, gave birth to a live child at term (P62).

The disappointing results obtained when progesterone with or without vitamin E and rest were prescribed are difficult to explain, especially as the pregnanediol values were exceedingly satisfactory (indeed high) in four patients: P11, P16, P50 and P53; of these P11 has already been described, and P53 miscarried after only a week's therapy. To P16 progesterone was

given intensively but only so long as the symptoms lasted (5 mg. daily for 5 days and 3 weeks in bed). Owing partly to the satisfactory figure for pregnanediol, therapy was then stopped, but abortion occurred 2½ months later. It is not known to what extent the patient followed instructions to rest, on being discharged.

The pregnanediol output in case P 50 was ascertained over a longer period and appeared satisfactory (see Fig. 6). Although during the 2 weeks before abortion the amount of progesterone had been reduced from 5 mg. twice weekly to 2 mg. thrice weekly, the amount administered during the preceding 8 weeks had been on a liberal scale and the reduction seemed justified. The fact that a high pregnanediol output actually coincided with abortion suggests that the latter may not have been due to a deficiency of progesterone. Other possibilities are discussed later, of which the most obvious is vitamin E deficiency. Although P 50 was receiving two capsules of vitamin E daily, this may have been inadequate, for it is known that individuals vary considerably in their ability to utilize vitamin E, as well as in their requirements. Shute [1939] is of the opinion that the latter increase as pregnancy advances. There is, besides, the doubtful factor of adequate rest being taken; the patient had already aborted twice, and rest was, therefore, imperative.

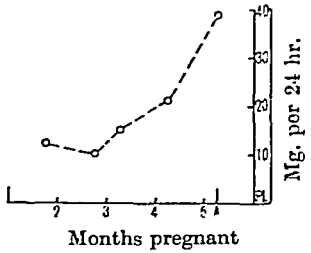


FIG. 6. Case P 50: the excretion of pregnanediol (*PL*). Abortion (A) at 21 weeks while receiving therapy.

With the exception of P 11 and P 50, therapy was either inadequate or started too late to prevent abortion.

Abortion associated with high values of pregnanediol

It was inevitable that some cases in which the patients aborted should be dealt with in the group devoted to threatened abortion. In seven of these the associated values of pregnanediol were definitely low, but in ten others abortion occurred at high or normal levels of pregnanediol output, and such cases (with two exceptions) are charted in Fig. 7. The two exceptions are P 50, shown separately for greater clearness (Fig. 6), and P 11, whose case has already been described (Fig. 3). The latter aborted 3 days after a high pregnanediol output, and again miscarried in similar circumstances when only 2½ months pregnant (as P 20, Fig. 7).

Including P 11 and P 50, there are seventeen women in whom abortion occurred within 1–14 days of a highly satisfactory output of pregnanediol. The time elapsing between the collection of the urine and abortion is given in Table IV.

It cannot be said that the abortion group contained a higher percentage of previous abortions than those of other groups in which live children were

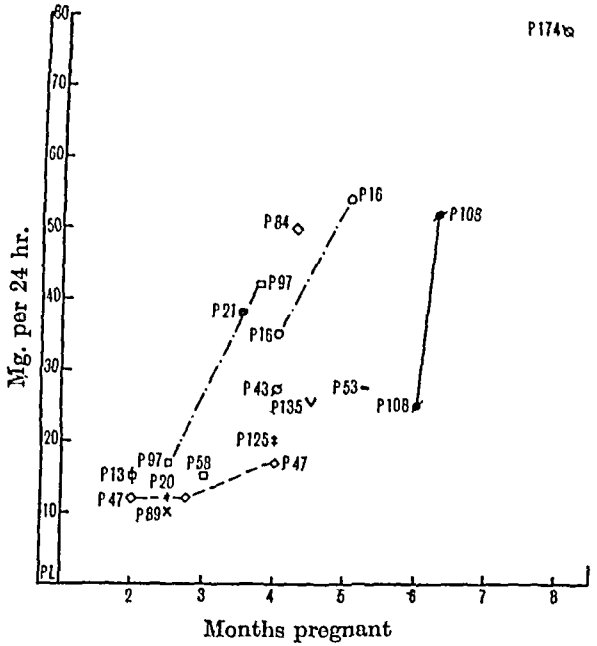


FIG. 7. Cases aborting at *high* levels of pregnanediol output (Tables IV and V, and Figs. 3 and 6).

Table IV. *Cases of abortion at high levels of pregnanediol output (Fig. 7)*

Case no.	No. of days between analysis and abortion	Case no.	No. of days between analysis and abortion
P11*	3	P58	10
P13*	10	P84†	7
P20	1	P89*	14
P16	11	P97*	14
P21	16	P125	2
P43	2	P135	12
P47*	2	P108‡	Same day
P50*	Same day	P174§	Next day (in labour
P53	4		at time of analysis)

* Receiving progesterone at the time (see Tables XV and XVI).
† A.Z. positive on day abortion occurred (foetus dead).
‡ Therapeutic abortion—cardiac case.
§ A.Z. weakly positive during labour (8 months).

born, as miscarriages had occurred in eleven out of seventeen patients aborting at high values contrasted with twenty-five[1] out of thirty-seven in

[1] Including P7, P8 and P40; P11 and P50 have been included in the abortion group.

the six normal groups, the percentages being 64·7 and 67·6 respectively. The number of previous miscarriages in the eleven patients is shown in Table V.

Table V. *The number of previous miscarriages in eleven women aborting at high levels of pregnanediol output*

1	2	3	4	5
P21	P47*	P11*	P20	P135
P43†	P89‡	P13‡	P97§	P174
—	P50‡	—	—	—

* Progesterone, vitamin E therapy and rest prescribed.
† Hydatidiform mole expelled in 1938.
‡ Progesterone and rest prescribed.
§ Progesterone and vitamin E therapy prescribed.

Complicating features, in addition to a history of previous miscarriage as listed in Table V, were as follows:

Hyperthyroidism: P50 and P97.

Symptoms of threatened abortion: P11, P16, P21, P43, P50, P53, P58, P84, P89, P125.

Albuminuria in this and the previous pregnancy: P13.

Marked hirsutism: P47. Dr Rosser (of Redhill) to whom I am indebted for this case writes: 'I have seen quite a few patients of this [masculine] type, all of whom have suffered these repeated disappointments.'

Progesterone therapy was actually being given at the time abortion occurred in seven patients, in four of whom (P11, 47, 50, 97) there was every reason to think that the amounts were adequate (see Table XV). In four others (P16, 53, 89, 174) progesterone was given over a brief period during which adverse symptoms were present. These became so severe in P53 that an attempt was made to induce labour but without success.

Condition of foetus and placenta. Reports on the products expelled were received in eleven cases: in seven of these (P47, 50, 53, 58, 108, 135, 174) the foetus was healthy and was either alive or had just died, and the placenta was normal in every respect. In the case of P174 (a funic presentation, already described on p. 26) death was due to obstetric causes which were not responsible for the abortion; the foetal heart was beating but breathing was not established. In spite of some hydramnios and a sudden increase in liquor amnii in P11's case, and severe retro-placental haemorrhage in P97, their foetuses were apparently normal. Although P84's foetus had been dead 2–3 days and some placental infarcts were present, gross abnormalities did not exist. This case is remarkable in that the Aschheim-Zondek test gave a standard positive reaction on the day that abortion occurred and when the foetus was already dead; the pregnanediol output 7 days before abortion was abnormally high for a 4½ months' pregnancy—50 mg./24 hr.

From the above it cannot be said that abortion was caused or generally accompanied by macroscopic abnormalities of the placenta or foetus, and the possibility of a derangement of function due to some undetermined cause has to be considered.

The author is indebted to Dr A. L. S. Macpherson[1] for P.108, a patient with cardiac disease in whom a therapeutic abortion was attempted on various occasions between the 2nd and 5th months of pregnancy. A living foetus weighing $1\frac{3}{4}$ lb. was born at $6\frac{1}{4}$ months, 5 weeks after the last oestrogen–quinine–castor-oil–pituitary induction, and a few hours after the high pregnanediol output charted. The circumstances are of special interest in that abortion was immediately preceded by a sudden increase in the output of pregnanediol; in one week this doubled itself—an increase of 26 mg. A direct connexion cannot be claimed between this phenomenon and a possible effect of the substances used for induction, as the interval that elapsed was too long (5 weeks). This does not, however, preclude the possibility of an indirect effect, as, for example, that an abnormal uterine condition was induced by the material injected, causing defective development of the placenta and that in time degenerative changes occurred which manifested themselves first in the outward symptoms of threatened abortion,[2] and finally in the release into the blood stream of all the pregnanediol stored in the placenta, possibly 2 or 3 days' supply. The effect of such a high elimination of progesterone would be to lower its concentration in the blood and to induce a condition which, conceivably, played an important part in abortion in the cases shown here, in all of which pregnanediol values were high. Presumably on those occasions on which a high pregnanediol output did not result in abortion (Fig. 10, Table VIII) there was no appreciable lowering of the concentration in the blood stream.

Another aspect involved is that of hormone utilization. The recovery of large amounts of progesterone in the form of pregnanediol glucuronide does not necessarily imply efficient utilization of the hormone (cf. p. 59), and it is possible that this utilization is closely linked up with factors connected with the process of hormone elimination. In this way it would still be possible to ascribe to progesterone the important role hitherto attributed to it in the maintenance of pregnancy. These alternative suggestions in explanation of a difficult problem are only tentative. It is clear that, in the cases belonging to this group, abortion does not simulate parturition, which is apparently associated with rhythmically declining hormone values.

The hypothesis that vitamin E deficiency may have been a contributory, if not a major, factor in bringing about abortion, is suggestive, especially as in only one case (P 97) can the amounts of vitamin E given be con-

[1] Fuller details of this case are being published by Dr Macpherson.

[2] In P 108 bleeding began 11 days before the abortion.

sidered adequate; in twelve patients vitamin E was not being administered. The occurrence of abortion in spite of high pregnanediol values becomes less puzzling if one bears in mind that progesterone does not counteract vitamin E deficiency [Drummond, 1939].

Abortion at low levels of pregnanediol output

On the assumption that parturition (and therefore abortion) is associated with, if not the result of, a drop in the secretion and excretion of progesterone, which is a theory commonly held, it would be expected that by

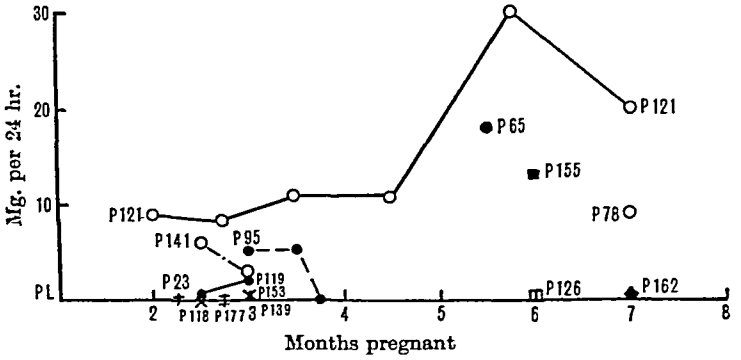


FIG. 8. Cases aborting at *low* levels of pregnanediol output (Table VI).

Table VI. *Abortion at low levels of pregnanediol output* (*Fig.* 8)

Case no.	Period between analysis and abortion
P23	Same day. [N.B. 2 previous miscarriages]
P65	14 days (alive)
P78	Same day (alive)
P95	Dead ovum; dilatation and curettage following day. [N.B. 5 previous miscarriages]
P118	7 days
P119	Dead ovum; dilatation and curettage following day
P121*	5 days (macerated 2 days). [N.B. 3 previous miscarriages]
P126	Next day
P139	Same day
P141	Day after 2nd specimen (dead foetus)
P153	Same day
P155	2 days (alive)
P162	Dead ovum; dilatation and curettage 3 days later
P177	3 days

* Patient aged 44; monster expelled at 7 months. Patient received 100 mg. progesterone in 20 weeks.

far the majority of abortions would belong to this group, yet it comprises only fourteen patients (Fig. 8, Table VI), of whom only three had had miscarriages already.

The interesting feature of these is that abortion was so long in occurring and that pregnancy was maintained in spite of an exceedingly low pregnanediol output: in eight patients abortion did not take place until after all trace of pregnanediol had disappeared from the urine, and in one other only 2·5 mg. were found the day before the patient aborted; in three patients in whom abortion occurred at or after 6 months (P65, P78 and P155) the foetuses were alive when expelled and equal, in development, to the stage of pregnancy, although the pregnanediol output was only 9–18 mg./24 hr. Yet we have already seen that pregnancy was not maintained in many women in whom large amounts of the glucuronide were being excreted. The logical conclusion is that progesterone is not the main factor responsible for the maintenance of pregnancy, but that abortion occurs independently of the amount of progesterone secreted and is due to a factor, or factors, which progesterone cannot inhibit. Supporting this argument is the fact that amounts of progesterone which should have been adequate to maintain pregnancy failed to prevent abortion.

A further anomaly exists in that the absence of pregnanediol excretion between the 3rd and 4th months does not necessarily indicate that pregnancy has ceased. This is dealt with later, but it appears to give added support to the suggestion that the maintenance of pregnancy does not depend on progesterone alone.

Pregnanediol values throughout pregnancy

Except on those occasions on which a clinician wishes to know merely whether sufficient pregnanediol is being excreted to make progesterone therapy worth while, a single analysis of pregnanediol output in the early stages of pregnancy is not very helpful, especially as abortions are not confined to the early months. In order to ascertain the trend throughout pregnancy, analyses should be made at regular intervals. Several authors [Venning, 1938; Browne, Henry & Venning, 1939; Bachman *et al.* 1940; Cope, 1940*a*] have given curves showing the extreme range of values within which pregnancy has followed a normal course, but in few instances has the course been followed (or shown) throughout individual pregnancies, though Browne *et al.* gave eight individual curves. The author has followed the pregnanediol output throughout a considerable number of pregnancies to ascertain if values may be related to various conditions or to specific stages of pregnancy, as well as to determine what can be considered abnormal and, as such, call for caution on the part of the physician.

In almost all the cases examined some anomaly existed: generally there was a history of previous miscarriage, still-birth or sterility; sometimes pregnancy was complicated by a fibroid of the uterus, or there was hyperemesis, or the uterus was large for the duration of amenorrhoea. In inter-

preting the curves, it is important to bear in mind that, in view of the number of abortions at satisfactory levels of pregnanediol output, one can never be sure that any value is safe.

The output of pregnanediol increases as pregnancy advances, and generally reaches a peak (or peaks) between the 7th and 9th months and subsequently falls. For the purposes of classification the following groups have been formed, based mainly on the maximum value of pregnanediol per 24 hr. attained: medium normals (50–70 mg.), medium high normals (70–90 mg.), low normals (30–50 mg.); three groups are devoted to an examination of low levels in early pregnancy; toxaemic and diabetic patients are dealt with separately. For details of therapy, reference should be made to Tables XV and XVI.

Medium normals (50–70 *mg.*), *Fig.* 9. Of twelve cases falling within this group, eight had had previous miscarriages, one as many as seven; where

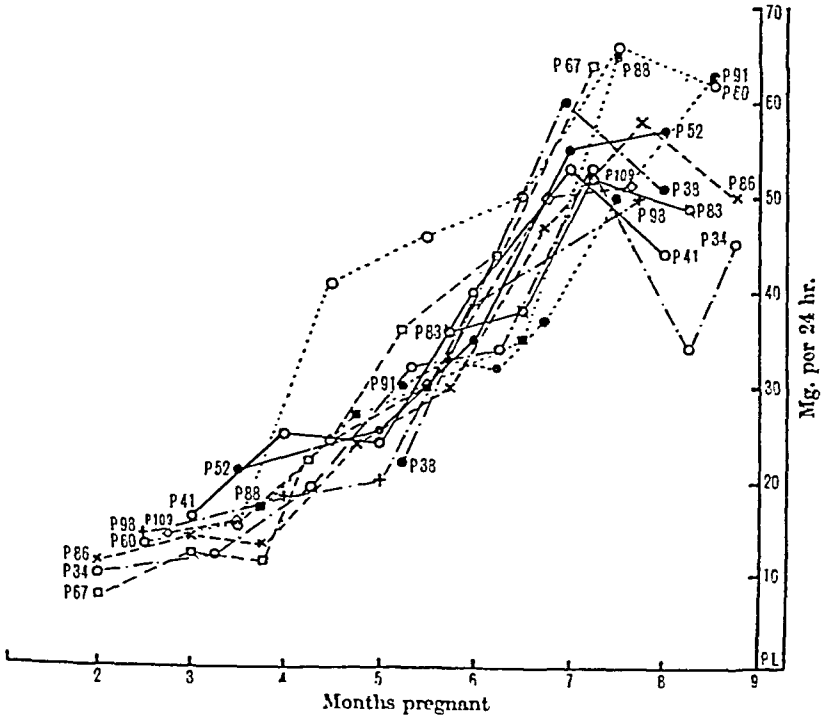


FIG. 9. Cases having a *medium normal* pregnanediol output (50–70 mg.) (Table VII).

known, the months at which these occurred have been indicated, also whether therapy was given (Table VII). In two cases analyses were asked for on account of haemorrhage, which was also experienced by two others of the series who had previously aborted. P86 is unique in having so little

Table VII. *Obstetric history of medium normals (50–70 mg. pregnanediol): twelve cases (Fig. 9)*

Case no.	No. of miscarriages	Haemorrhage month	Therapy
P38	7 1 at 3½ months 6 at 6–7½ months	None	Progesterone
P41*	1 at 3 months	3–3½	Progesterone, vitamin E, rest
P60	1 at 3 months	None	Vitamin E
P67	1 ? stage of pregnancy	None	Progesterone
P83	2 at 6 months	None	Progesterone
P88	1 at 5 months	None	Progesterone
P91	2 at 6–7 months	None	Progesterone, vitamin E, rest
P98	1 at 3 months	2–3	Vitamin E
P109*	2 still-births	None	None
P34	None	2	None
P52†	None	2½–3½	None
P86‡	None	None	Progesterone, vitamin E

* By Caesarian section. † No child for 9 years. ‡ No child for 8 years.

ovarian tissue; after a right salpingo-oöphorectomy and resection of the cystic portion of the left ovary by Dr Sturrock in 1936, a live child was born to the present pregnancy in 1941 after 8 years of married life. Live children were born to all members of this group; in two patients Caesarian section was performed—due to a breech presentation in P41, and to foetal deaths 3 weeks before delivery in previous pregnancies in the case of P109, who also had renal complications.

The following points characterize this group: The pregnanediol output was almost stationary round about 10 mg. from the 2nd to the 4th months. A peak was reached at approximately 7 months, followed by a drop at 8–8½ months with a subsequent rise, in some cases, before parturition. The output of pregnanediol during haemorrhage was highly satisfactory in all cases.

Medium high normals (70–90 *mg.*), *Fig.* 10. The feature held in common by the five cases comprising this small group (Table VIII), to all of whom live children were born, is the marked increase in the amount of pregnanediol excreted at 5½–6 months. So sudden a rise is, as we have seen, suggestive of imminent abortion; it is also often associated with a toxic condition, but it is clear that it does not necessarily denote that such exists or is likely to develop. It is possible that in such cases foetal development is rapid in the early stages, seeing that, in two instances, the uterus was larger than the period of amenorrhoea justified and complications were suspected. The occurrence of hydatidiform moles in the two previous pregnancies in the case of P85, causes one to speculate as to the possibility of a third, had the marked secretory activity at 5¾ months occurred instead at 2–3 months.

Table VIII. *Medium high normals* (70–90 *mg. peak*) (*Fig.* 10)

Case no.	No. of miscarriages	Haemorrhage month	Therapy
P56*	None	3–3½	Vitamin E till 5 months
P80*	None	None	None
P85	2 hydatid moles at 3 months	None	Vitamin E till end of 7 months
P94	2 at 7 months—not viable	None	Progesterone
P120	3 at 6–12 weeks	2–3	Vitamin E till 8 months

* Uterus large for dates. Fibroids or hydatidiform mole suspected.

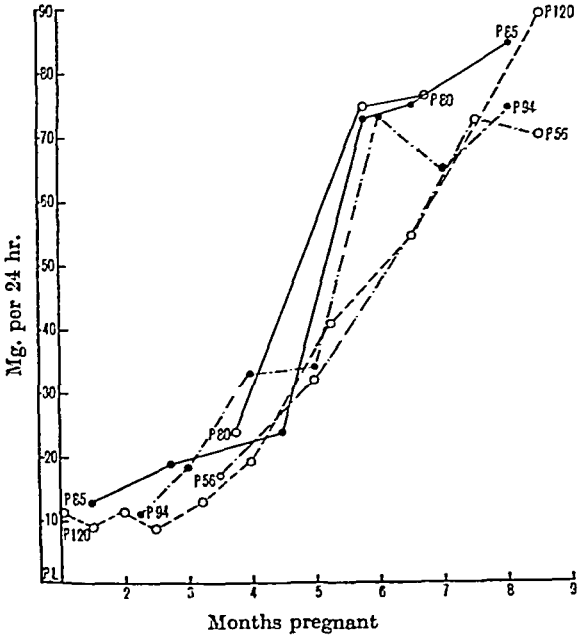


FIG. 10. Cases having a *medium high normal* pregnanediol output (70–90 mg.) (Table VIII).

Low normals (30–50 *mg.*), *Fig.* 11. All seven cases gave birth to live children at term, although Caesarian section was performed in one instance owing to a breech presentation (P31). Four were not submitted for analyses until after the 5th month (see Table X).

In spite of the low pregnanediol output in these cases, in only one instance did the child weigh less than 6 lb. at birth. This fact suggests that a relatively low secretion of progesterone is sufficient both for the maintenance of pregnancy and for normal foetal development, a statement which makes the abortions at high values still more incomprehensible.

The existence of a low pregnanediol output in a case of twins might be explained by the greater demands made on the mother of two foetuses.

It will be observed that hyperemesis gravidarum was associated with low pregnanediol outputs in three women, although at the time of the symptoms the output was generally satisfactory.

The possible significance of the drop in output which occurred in P22 and P25 between the 3rd and 4th months will be discussed later.

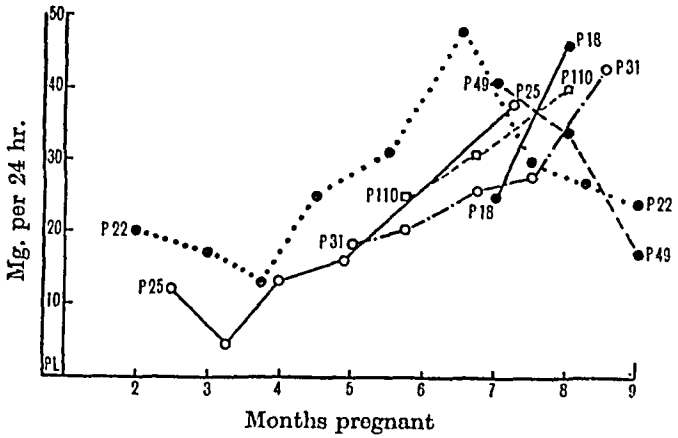


FIG. 11. Cases having a *low normal* pregnanediol output (30–50 mg.) (Table IX).

Table IX. *Low normals* (30–50 *mg.*): *seven cases*

Case no.	No. of miscarriages	Haemorrhage month	Therapy	Remarks
P18	4 (6–7 months)	None	Vitamin E, rest	Age 39; live child full term
P22	None	None	None; diet unrestricted	Uterus very large; fibroid, hyperemesis, phlebitis. Child $6\frac{3}{16}$ lb.
P31	2 (5–6 months)	None	Progesterone, vitamin E, rest	Age 34; 11 years married, marked anaemia at 31 weeks, child $6\frac{3}{16}$ lb.
P49	None	None	None	Weight increased 1 stone in $2\frac{1}{2}$ months; twins 6 and $5\frac{1}{16}$ lb.
P110	3 (? months)	None	Progesterone	Live child full term
P25	3 (? months)	None	Progesterone	Hyperemesis. Child $5\frac{9}{16}$ lb.
P124	2 (2–3 months)	2–3	?	Hyperemesis. Pregnancy still running (7–8 months)

Low levels in early pregnancy. Whereas in many pregnant women the output of pregnanediol remains almost stationary in the neighbourhood of 10 mg. until about the 4th month, others show a drop of varying degree between the 3rd and 5th months, preceded as a rule by normal or relatively high values. Such cases fall into three groups according to the nature of the drop (Table X, Figs. 12–14). The special interest that this fall in output has, apart from the anxiety that it is apt to cause, is its possible relation to the establishment of placental function and the withdrawal of ovarian secretion. In most cases the transition is likely to be gradual; it may sometimes happen, however, that placental activity is late in being

established or that ovarian secretion has been slow in diminishing. The high values which generally precede the fall in pregnanediol output might be explained as due to ovarian + placental function, the ovarian activity being very marked, and the drop[1] as being caused by partial withdrawal of ovarian function. In the cases charted in Fig. 12 this would, presumably,

Table X. *Cases showing fall in pregnanediol output in early pregnancy (Figs. 12–14)*

e no.	Miscarriages	Haemorrhage	Therapy	Outcome and remarks
12.	*Fall at 3–4 months*			
14	1 (3 months)	Loss during most of pregnancy, especially last month	Progesterone, vitamin E, rest	Married 5 years: child $6\frac{2}{16}$ lb.
22	None	None	None	Fibroid, hyperemesis, phlebitis: child $6\frac{3}{16}$ lb. [see low normals]
25	3	None	Progesterone	Hyperemesis; child $5\frac{10}{16}$ lb. at $8\frac{1}{4}$ months [see low normals]
96	1 (4 months)	None	Progesterone	Live child at $8\frac{1}{2}$ months
123	None	Bleeding on effort	Vitamin E, rest	Live child at 9 months
13.	*Fall at 4–5 months*			
26	3 (2–3 months)	None	Vitamin E	Aged 41; married 19 years; child $7\frac{1}{2}$ lb.
'27	None	2–3 months	Progesterone, vitamin E, rest	Child $8\frac{1}{4}$ lb.
28	None	2–3 months	Progesterone, vitamin E, rest	Child $7\frac{6}{16}$ lb.
'57	1 still-birth; 1 abortion $7\frac{1}{2}$ months	None	Progesterone, vitamin E	Child $7\frac{6}{16}$ lb. (Caesarian)
. 14.	*Nil at 2½–4 months*			
'51	None	None	None	Hypopituitarism; little increase in size of uterus till $4\frac{1}{2}$ months; bronchiectasis; induction; child $6\frac{3}{16}$ lb.
'62	None	1–2 months	None	? Ectopic; pelvic inflammation; child $7\frac{5}{16}$ lb.
'77	1 ($2\frac{1}{2}$ months)	None	None	Child $7\frac{3}{4}$ lb.
'93	3 (3 months)	None	Progesterone, vitamin E, rest	Child $5\frac{5}{16}$ lb. (premature: $7\frac{1}{2}$ months)

diminish between 3 and $3\frac{1}{2}$ months, whereas in those belonging to Fig. 13 it must have lasted until 4 months. In the third group (Fig. 14) consisting of four women in whom the merest trace of pregnanediol (1 mg. or less) was found, there would seem to have been a hiatus between the withdrawal of ovarian activity and the establishment of placental function. This was observed at various points between $2\frac{3}{4}$ and $3\frac{3}{4}$ months and, in one

[1] In the second group this drop is to normal values.

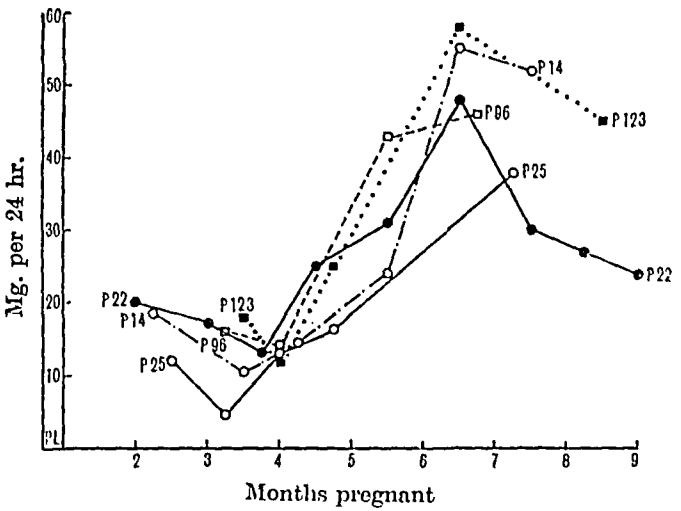


FIG. 12. Cases showing fall in pregnanediol output at 3–4 months (Table X).

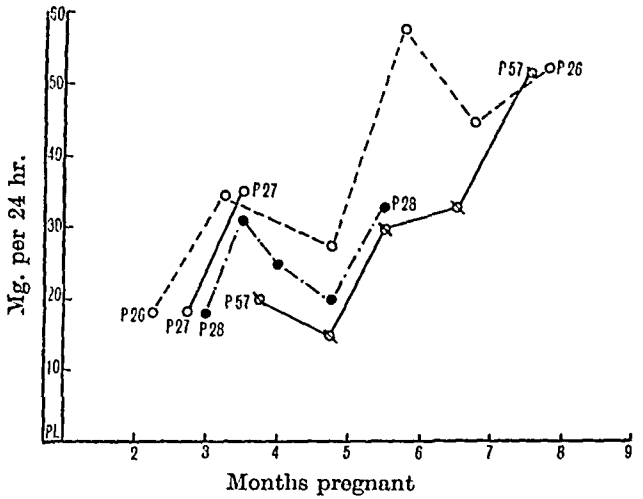


FIG. 13. Cases showing fall in pregnanediol output at 4–5 months (Table X).

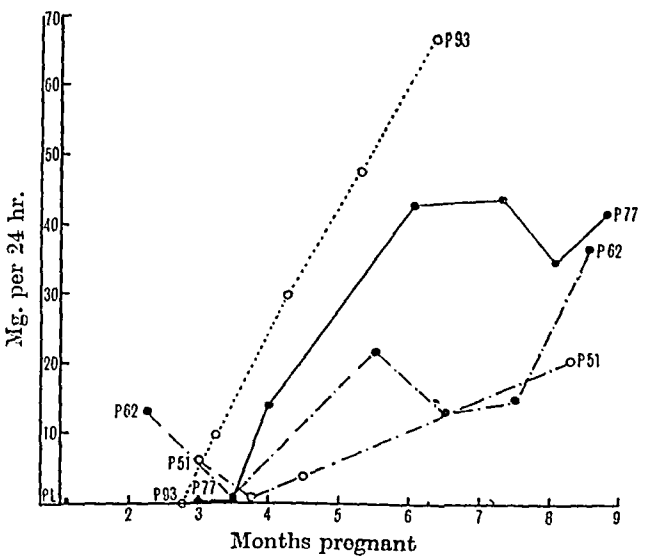


FIG. 14. Pregnanediol output *nil* or 'only a trace' at 3–4 months (Table X).

instance (P77), on two occasions separated by a fortnight. In all patients, with the exception of P51, pregnanediol values rose rapidly, suggesting that its absence from the urine might have been due to its retention within the body owing to a faulty formation of the glucuronide, rather than to an hiatus in its production. Browne *et al.* [1939] have reported a similar occurrence at the 49th day, and Cope [1940*a*] one as late as the 19th and 21st weeks of pregnancy, associated with symptoms of threatened abortion. The latter case recalls that of P40 (p. 22), in whom only a trace of pregnanediol was found at 6¼ months; both Cope's patient and P40 gave birth to live children as did also all those belonging to the three groups just described.

The necessity for caution in interpreting a negative pregnanediol result is obvious, and it has become our practice, in such circumstances, to ask for an Aschheim-Zondek test to follow; when symptoms of threatened abortion are associated, the prognosis is generally bad. The low levels of pregnanediol excretion in early pregnancy in this series cannot be attributed to symptoms of threatened abortion as in few cases were these present, and notably in two—P27 and P28 (Fig. 13)—the output was high throughout the time that the symptoms were manifest.

Particulars of the patients comprising the three groups are given briefly in Table X. Two cases in the first section (P22 and P25) have been dealt with under 'low normals'. In spite of therapy and abundant rest throughout pregnancy, P14 had a persistent, blood-stained discharge which became more pronounced during the last month. Her pregnanediol values were good and pregnancy terminated successfully. In the second group P26 is outstanding as, aged 41, after three miscarriages and 19 years of married life, she had a live child after treatment with vitamin E; owing to her satisfactory pregnanediol output progesterone was not given.

The maximum values reached in the three groups tended to be low; especially was this the case in P51 (Fig. 14, group 3) who excreted only 20 mg. pregnanediol at 8¼ months (cf. normal 40 mg. or upwards). In spite of an unsatisfactory clinical condition, a live child was born. The long interval between the last two analyses was due to the fact that it was thought that foetal death had occurred.

The case of P62 is abnormal, not only on account of the absence of pregnanediol at 3½ months but also because of the low level attained between the 6th and 8th months. A similar drop occurred in P40 (p. 22), and its significance is not understood.

It is obvious from the average weight at birth that the fall in pregnanediol output in the early stages of pregnancy had no adverse effect on the subsequent course or outcome of pregnancy.

The pregnanediol excretion has also been ascertained in the following conditions: toxaemia, diabetes and foetal death.

Pregnanediol output in toxaemia

Both Weil [1938] and Browne *et al.* [1938] found that, in late toxaemia of pregnancy, pregnanediol was either absent or excreted in very small amounts. Cope [1940*b*], in a study of ten cases, recovered normal amounts of pregnanediol and suggested that when its excretion is greatly reduced

Table XI. *Toxaemia of*

Case no.	Miscarriages	Age	Blood pressure	Albumen in urine	Oedema
P12	Para 0	37	?	?	?
P13	1 at 2½ m. 2 still-born	36	180/100	Urine 'loaded' at first analysis	?
P36	2 at 7 and 11 w.	30	150/90	0·05 %	In both legs
P46	2 at 3–4 m. 6 para	44	150/90 at 5 m. 200/110 at 7¼ m. 220/150 at 8¼ m.	Trace	Ankles only
P45	?	41	?	Present	?
P68	5 (various) 14 para	42	?	?	?
P74	Para 0	33	170/96 at 5 m. 140/90 at 7 m.	Trace	None
P99	2 still-born (1931, 1933)	25	170/106	500 mg./l.	+
P100	Para 0	33	145/100	None	None
P102	Para 0	24	150/100	2 g./l.	++
P103	Para 0	28	180/120	1¼ g./l.	None
P104	Para 0	25	160/120	1 g./l.	++
P105	1 still-born (anencephalic)	34	202/100	'Solid'	Severe in legs, headaches
P116	Para 0	21	?	'Almost solid'	++
P109	2 still-born	33	Max. 150/100	1 g./l.	Ankles and face

m. = months, w. = weeks of pregnancy.

in toxaemia, this may be due to a complicating chronic nephritis resulting in renal damage. In a single instance of severe eclampsia the author recovered the largest amount of pregnanediol so far recorded on a single day (274 mg.) [Hain, 1940].

In the fifteen cases comprising this group (Table XI, Fig. 15), the following values obtained at 30–39 weeks, 77, 64, 55, 52, 51, 50, 37, 35, 30, 16, show a tendency towards a high output rather than a low one. In only

one of these cases can the output be described as low; low values were also obtained in two other patients who did not go beyond early pregnancy.

There was a history of toxaemia in previous pregnancies in three cases (P13, P68, P99) of whom P68 had had five abortions from this cause in fourteen pregnancies in as many years. Owing to her history and chronic

egnancy (*Fig.* 15)

Urea nitrogen	Child	A.Z.*	Remarks
?	Still-born at 7¾ m.	+ at 6¾ m.	Acute toxaemia 3 weeks before analysis induction 6 weeks later; 'foetus dead considerable time, probably 6 weeks'
?	Miscar. at 2½ m.	+ at 5½ w.	Eclampsia in last pregnancy (1939) when macerated foetus born at 8 m.
?	Live, full term	+ at 5 w.	Toxaemia developed at 36 weeks, responded to rest in bed. Child 7 1/16 lb.
ood urea 46 mg. day after delivery	Live at 8¼ m. (3¾ lb., survived)	++ at 5 m.	At 5 m. uterus = 7 m. but no foetal heart felt; slight toxaemia at 5 m. responded to rest; medical induction at 8¼ m. because of blood pressure
?	Live at 7¾ m.	+ at 6¾ m.	Abortion (= 7 m. development)
?	Pregnancy interrupted	+ at 6 w.	14 pregnancies 1916–40 of which five abortions; toxaemia with most of her pregnancies; chronic nephritis; sterilized at 2½ m.
?	Miscar. at 7 m.	++ 1/10 + 1/100 − at 5 m.	Bleeding during 2nd and 3rd m.: antepartum haemorrhage due to toxaemia and placenta praevia
ood urea 10 %	Live at 8 m.	Not done	Kidney trouble from 1933 (last child first child spina bifida; delivery c after analysis
lood urea 10 %	Live at 9 m.	Not done	Membranes ruptured
.N. 12 mg. %	Live at 8¾ m.	Not done	Twins: medical induction
.N. 14 mg. %	Live at 8½ m.	Not done	Child weighed 4¾ lb.; medical induction
.N. 17 mg. %	Live at 8½ m.	Not done	Child weighed 7½ lb.; medical induction
.P.N. 37 mg. %	Live at 7¾ m.	Not done	Retro-placental haemorrhage; numer infarcts. No pregnancy for 11 years; ch 5 11/16 lb. (died)
?	Not viable	Not done	Uterus emptied same day as analysis d (22 weeks)
?	Live at 9 m.	Not done	By Caesarian section: blood press 150/100 day before

++ = Standard positive, + = weak positive, − = negative.

nephritis she was sterilized at 2½ months. Of the fifteen women, ten were over 30 years of age, of whom only five had viable children in the present pregnancy; of these P46 is outstanding as she gave birth to a 3¾ lb. child which survived. In all, nine had viable offspring, four aborted and in two pregnancy had to be interrupted; in several instances labour was induced owing to an unsatisfactory clinical condition, and there was one Caesarian section owing to high blood pressure.

There was a previous history of miscarriage or still-birth in at least six cases, two of the patients (P13, P36) receiving progesterone therapy on this account, in spite of which P13 again miscarried. P36 was given 5 mg. progesterone weekly from the 6th to the 10th weeks only, and thereafter vitamin E, adexolin and calcium lactate until 7½ months, when she developed mild toxaemia; this responded to rest in bed and pregnancy terminated successfully. The onset of symptoms of toxaemia coincided with a pronounced rise in pregnanediol output, which, however, did not reach excessive values. It is of interest that the administration of progesterone and vitamins in the early stages of pregnancy did not prevent

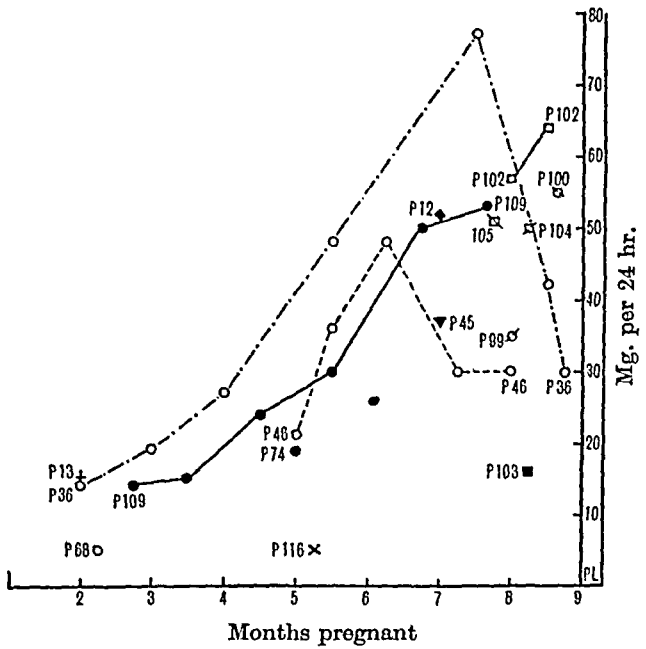


FIG. 15. *Toxaemias of pregnancy*—pregnanediol output (Table XI).

the development of mild toxaemia at a later stage, and also that pregnanediol values before its onset were quite normal; this normality was observed also in P46 and P109 who was not treated other than by rest.

It was first reported by Smith & Smith [1934] and later confirmed by Rakoff [1939] and by Siegler [1939] that the gonadotrophin content of the urine and/or serum in cases of pregnancy toxaemia was high, but Browne *et al.* [1938] were unable to confirm these findings. In the present series, the evidence in six patients in whom the gonadotrophin output is nearly related in time to the onset of the symptoms of toxaemia, supports Browne *et al.*, the Ascheim-Zondek tests given in Table XI recording weak positives in four (P12, P13, P45, P68) and standard positives in two others (P46, P74).

Pregnanediol output in diabetes

The pregnanediol output was also ascertained in three diabetic patients (Table XII, Fig. 16); two of these were diabetics of 5 years' standing, whereas the condition had developed only 3 months before the present pregnancy in the third (P69) who had had two abortions. There was a sudden onset of toxaemia at 8 and 7¾ months respectively in the first two (P64 and P76); this was accompanied by a sudden increase in pregnanediol output, which was very marked in P64, in whom it rose in one month from 64 to 137 mg./24 hr. At the time of the last analysis in P76 albuminuria had not developed, but oedema was present and the figure for pregnanediol had already reached 90 mg. at 7¾ months. The eclamptic convulsions and hypoglycaemic attacks which occurred in this patient during labour are to form the subject of a report by Dr C. E. Peaker to which reference may be made. In both cases pregnancy was terminated by Caesarian section.

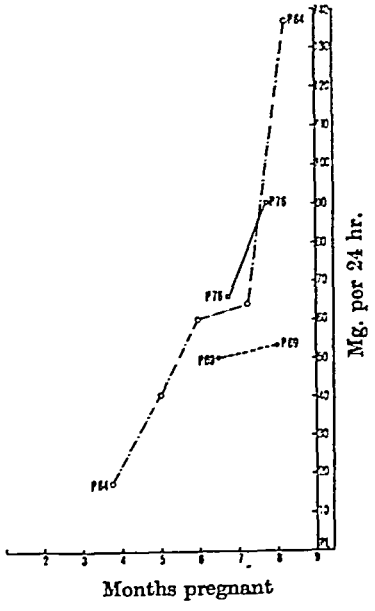


FIG. 16. Pregnanediol output in *diabetes* (+toxaemia in P64 and P76) (Table XII).

The occurrence of a marked increase in pregnanediol excretion at the onset of symptoms of toxaemia has caused the writer to view sudden increases with some suspicion. It will be recalled that the group of abortions at high pregnanediol values contained several such cases; however, the group of medium high normals contains three outstanding examples of this type in all of whom pregnancy was uneventful.

Pregnanediol output in cases of foetal death

This group (Table XIII) comprises thirteen cases in which the foetus was already dead at the time that the pregnanediol output was ascertained, and two others in which foetal death occurred 3 and 7 days afterwards. These two (P84, P107) have been included, as it was possible to relate the values very closely to the time at which the foetus died; in both cases these values were high, but abnormally so in P84 who was excreting 50 mg. at 4½ months.

Of the fifteen patients, only four aborted, and these have been described in the section devoted to abortion. In a case of ectopic pregnancy (P66), the foetus was removed after the patient's death. A hydatidiform mole was

Case no.	Age	Abortions	Therapy	Toxaemia	Diabetes	Outcome
P64	45	1933: induction because of oedema and albumen; para 4	Progesterone 1 week only (15 mg.) at 3½ months	Oedema and hypertension; albumen + + 1 week before induction; blood pressure 145/100 at delivery	Diabetes mellitus since 1935; obese: 14 st. at conception, 16 st. at delivery, 1940	Hydramnios, transverse lie; Caesarian section at 8 months because of toxaemia; ♂ 13 lb.
P76	27	Para 0	Vitamin E only	Albuminuria, oedema, hypertension, blood pressure 150–180/100, convulsions, obesity, severe headaches	Diabetes 5 years glycosuria and raised blood sugar; hypoglycaemic convulsions after insulin	Medical induction followed by Caesarian section when albumen + + +, blood pressure 180/100 and gross oedema. Live child 10 11/16 lb. at 8¼ months
69	38	2 still-births at 7 months (1928, 1930)—latter a hydramnios	From 5 months vitamins A and E and calcium lactate daily	No albumen, no oedema, blood pressure 130–145	Diabetes since 1939 (Aug.); mild glucosuria, raised blood sugar, responded to diet	Live child 5 7/16 lb. at 8½ months

Table XIII. *Pregnanediol output in cases of foetal death*

Case no.	Analysis done at months	Foetus dead for	Pregnanediol/24 hr. mg.	Accompanying condition	Remarks
P12	7	3 weeks	52	Acute toxaemia	Labour induced
P107	6½	—	42	Pyrexia, acute hydramnios	Labour induced
P71	6	>4 weeks	54	Severe hydramnios; ovarian serous cyst	Labour induced; anencephalic monster
P113	6	A few days	28	Transverse myelitis, and complete paresis of lower limbs	Labour induced
P126	6	?	Only a trace	None stated	Abortion day after analysis
P116	5¼	'Recently'	5	Severe toxaemia	Uterus emptied same day as analysis done
P66	5	Ectopic	9	Fibroids, pyrexia, placenta in mass of adhesions to intestines	Patient died 3 days after analysis: post-mortem report
P84	4¼	—	50	Intermittent loss 2 months; placental infarcts	Analysis done 3 days before foetus died and 1 week before abortion
P95	3–3¾	?2–3 weeks	(*a*) 5 at 3 months	—	Five previous miscarriages; dilatation and curettage done
			(*b*) 5 at 3½ months		
P75	3	?	(*c*) Nil at 3¾ months	Hydatidiform mole	Severe haemorrhage 'for some weeks'
P153	3	?	5·5	Considerable haemorrhage	Aborted day of analysis
P141	3 and 2½	?	A trace	Considerable haemorrhage	Aborted day after 2nd analysis
P118	2¾ and 2½	? >1 week	(*a*) 5·6; (*b*) 2·5	Severe hyperemesis	Haemorrhage started 3 days after 2nd analysis
P150	1½	?	A trace both times	Intermittent bleeding since conception	Dilatation and curettage done
P119	2½–3	?	A trace	Severe haemorrhage for 8 days	Dilatation and curettage done
			Hardly a trace		

expelled by P75 and nine other patients had medical inductions or the uterus was emptied.

In seven cases foetal death occurred at or before the 3rd month, and, as was to be expected at this early stage, the pregnanediol output was either very low or negligible. When foetal death occurred after the 5th month, the values were generally high (five cases); however, in two cases at 5 months and one at 6 months very low values were obtained (P66, P116, P126).

We have already seen that a satisfactory pregnanediol output is no guarantee that abortion will not occur, and it is also clear that such values do not necessarily indicate that the foetus is alive. There is nothing in the attendant circumstances that might account for the totally different values associated with foetal death, and it is of interest that, in two women with severe or acute toxaemia, 5 and 52 mg. pregnanediol were excreted respectively at $5\frac{1}{4}$ and 7 months (P116, P12), although in the latter the foetus had been dead for 3 weeks. It can generally be said that values as low as those found in P66 and P116 (Table XIII) at and after the 5th month are strongly suggestive of foetal death, but one cannot be certain in view of the very low output in P40 at $6\frac{1}{4}$ months (p. 22) and the absence of pregnanediol at 19 and 21 weeks in a case recorded by Cope [1940*a*].

Therapy

In an investigation of the effect of progesterone therapy in cases of recurrent abortion, MacGregor & Stewart [1939] describe the results of treatment in twenty patients who had had two or more successive abortions. The percentage of live births was 64, a figure significantly above the

Table XIV. *Table showing outcome of pregnancy ± therapy*

Category	No. of cases	Failures %	Successes %
(*a*) Those with previous miscarriages	55	36·4	63·6
(*b*) Those with 2 or more miscarriages	39	41	59
Of (*b*) receiving progesterone ± vitamin E	23	39	61
Of (*b*) receiving vitamin E only	7	14	86
Of (*b*) receiving no therapy	9	66·6	33·3
All receiving progesterone ± vitamin E	34	26·5	73·5
All receiving vitamin E only	13	15·4	84·6
All receiving no therapy*	54	60	40

* Including some that had therapy for 10 days or less (Table XVII).

40 % to be expected on the calculations of Malpas [1938] when the number of previous abortions was more often three than two. In our series of twenty-three cases having a history of two to nine previous abortions (Tables XIV and XV) nine aborted and fourteen had live children, successes after progesterone therapy being 61 %. As twelve of the twenty-three women had three or more abortions the expectation of a full-time pregnancy would be

Table XV. *Particulars of patients receiving progesterone ± vitamin E therapy*

Case no.	No. of miscarriages	Rest*	Progesterone total mg.	Stage at which progesterone given	Vitamin E capsules	Outcome of pregnancy
P11	3	+	83†	16th–22nd week	3 daily throughout	Abortion at 22nd week
P13	3	?	?	?	?	Abortion at 10th week
P47	2	+	60	5th–16th week	1 daily from 5th week	Abortion at 4 months
P50	2	++	134	5th–20th week	2 daily from 5th week	Abortion at 21 weeks
P61	3	?	?	3rd–18th week	? amount from 5th to 7th month	Abortion at 7 months
P89	2	++	14	7th–13th week	3 daily from 7th week	Abortion at 13 weeks
P97	4	—	55	10th–18th week	3 daily from 10th week	Abortion at 18 weeks
P101	8 or 9	?	82	6th–11th week	2 daily from 6th week	Abortion at 11 weeks
P121	3	++	100	12th–31st week	1 daily for first 6 weeks	Abortion at 7 months
P88	1	+	52	19th–31st week	None	Full term; death during difficult delivery;‡ breech
P115	2	+	28	24th–31st week	None	Full term;‡ occipito-posterior
P7	2	++	200	4th–37th week	300 throughout pregnancy	Live
P8	3	+++	215	8th–30th week	None	Live
P14	1	+++	116	8th–32nd week	3 daily throughout	Live
P25	3	+	32	23rd–31st week	None	Live
P27	0	+	25	9th–11th week	9 daily first 2 months	Live
P28	0	++	25	10th–14th week	3 daily throughout	Live
P31	2	+++	100	24th–37th week	2 daily from 16th week	Live; breech; Caesarian
P36	2	++	25	6th–10th week	3 daily throughout	Live
P38	7	++	210	18th–39th week	3 daily from 18th week	Live
P40	1	+	190	13th–32nd week	3 daily throughout	Live
P41	1	++	24	10th–14th week	3 daily throughout	Live; breech; Caesarian
P57	2	++	206	14th–37th week	2 daily throughout	Live; breech: Caesarian
P64	1§	+	15	15th–17th week	None	Live; breech; Caesarian
P67	1	+	60	9th–22nd week	None	Live
P83	2	+	20	27th–31st week	None	Live
P86	0	+	230	10th–32nd week	1 daily throughout	Live
P91	2	++	130	24th–38th week	3 daily 20th–28th week	Live
P93	3	+	240	12th–25th week	2 daily 12th–25th week	Live (premature: 7½ months)
P94	2	+	56	18th–31st week	None	Live
P96	1	+	32	24th–31st week	None	Live
P110	3	+	80	25th–30th week	None	Live
P111	0	+	55	21st–32nd week	1 daily from 3rd month	Live (deformed)
P159	4	+++	30	27th–29th week	1 daily from 23rd week	Live

* Rest: + In bed during symptoms of threatened abortion or monthly or occasionally. ++ In bed 2 months, or 1–2 months plus daily rest over long perid. +++ In bed 3 months or more, plus daily rest.
† Including 35 units during the 24 hr. preceding abortion, but excluding progesterone received before 4th month.
‡ Counted as successes.
§ Induction, because of toxaemia.

the same as MacGregor & Stewart's, that is 40 %,[1] and it is with this figure that our 61 % should be compared. The results are thus mainly in agreement with those of MacGregor & Stewart [1939] and of Bishop [1937], Kane [1936] and Elden [1938] in supporting progesterone therapy.

Reference to Table XIV brings out three interesting facts: (*a*) The percentages of failures and successes after two or more miscarriages are not significantly altered if the totals include those receiving either vitamin E therapy or no therapy at all (Table XVII) or if those with only one previous

Table XVI. *Particulars of patients receiving vitamin E therapy*

Case no.	No. of miscarriages	Rest	Vitamin E capsules	Outcome of pregnancy
P17	4*	+	30 mg. daily for 10 days at 4th month	Still-birth—incomplete abortion at 7½ m.
P84	0	+++	1 daily from 8th week	Abortion at 18th week
P18	4*	+++	? Amount (E, A and D) throughout pregnancy	Live
P26	3†	+	2 daily throughout	Live
P56	0	+	*c.* 80 capsules, 3rd–5th months	Live
P60	1	–	1 daily from 3rd month	Live
P69	2	–	1 daily from 5th month	Live
P76	0	–	1 daily throughout pregnancy	Live: Caesarian at 8¼ m.
P98	1	+	2 daily from 2½ months	Live
Dr R's X	4‡	++	2 daily from 8th to 13th week 9 daily from 14th week on	Live
P85	2§	–	2 daily from 6th to 31st week	Live
P120	3‖	++	2400 mg. from 4th to 24th week	Live
P123	0	++	3 daily from 8th to 16th week	Live

* All at 6–7 months. † All at 2–3 months.
‡ Two at 2–3 months, two at 4 months (no pregnanediol analyses).
§ Both hydatidiform moles. ‖ At 6–12 weeks.

Table XVII. *Particulars of fifty-four patients receiving no therapy*

Category	Total no. of cases	Stage at which miscarried or aborted in this pregnancy: no. of cases						Foetal death 6½–7½ months
		2–4 months		4¼–6 months		6½–8 months		
		15		9		6		2
		Previous miscarriages						
		1	2	3	4	5	6	Twins
Abortions or miscarriages	32*	4	1	1	1	3	—	
Live offspring	22†	3	2	—	—	—	—	3

* Three toxaemias of pregnancy. † Six toxaemias of pregnancy.

miscarriage are added. (*b*) The group of patients with a history of two or more abortions given vitamin E therapy alone, although small (only seven cases, Tables XIV and XVI), contains three women with four previous

[1] Chance of pregnancy after > four abortions assumed = chance after four only.

miscarriages, and for this reason the 86 % successes may be considered significant. Especially is this the case when one compares this with only 33·3 % successes when no therapy was given to a group of similar size. The number of cases is too small to afford a proper comparison with the results of others, but it is noteworthy that the records of some hundreds of cases of habitual abortion treated with vitamin E give a mean of 75–80 % favourable results. The literature is fully discussed by Vogt-Möller [1939]. The data in Table XVI do not seem to support Shute's contention [1935, 1936, 1937] that its beneficial value is limited to cases of threatened abortion, and that those patients habitually aborting do not respond. (*c*) It is possible to compare the merits of various kinds of therapy only when these do not overlap. It was not intended at the outset of this study that such a comparison should be made, but since the therapy given was bound to affect the outcome of pregnancy, an effort has been made to evaluate this effect. Three forms of treatment have been considered: progesterone, vitamin E, and rest. For the last-named an arbitrary standard has been adopted as described at the foot of Table XV. *The effect of rest must be allowed for in assessing all forms of therapy, as also the part played by its absence when therapy was unsuccessful.* In the group devoted to vitamin E (Table XVI) rest may have played an important part in the outcome in P18 and P26 but it failed to compensate for inadequate vitamin E therapy in P84. Although P26 did not take any period of prolonged rest, it is likely that the abstention from effort played a considerable part in achieving success, as all previous pregnancies had been associated with bank removals (her husband was a banker).

The difficulty of assessing the effect of therapy between progesterone and vitamin E when both were given is obvious; the effect of rest has to be considered here also (Table XV). Examination of the cases in which the patients aborted suggests that in two (P11, P97) both forms of therapy were adequate, but insufficient rest was taken. The data are incomplete for P13 and P61, but vitamin E was certainly inadequate in P47's case and may have been in P50's.

There has been much speculation as to the mode by which vitamin E acts, whether via the pituitary or directly on the gonad. Vogt-Möller [1936] considers that the collaboration of vitamin E with chorionic gonadotrophin is necessary for the formation of corpus luteum hormone, since Nielsen [1935] showed that the luteinization which occurs in rabbits' ovaries in a positive Friedman test is dependent on the vitamin E which the rabbit receives in its food. Nielsen states, moreover, that the cholesterol content of the blood, which is high in normal pregnancy, but low in cases of habitual abortion, is raised by vitamin E. According to Shute [1936] liberation of an excess of oestrogens may be taken as evidence of vitamin E

deficiency; in other words, vitamin E is anti-oestrogenic. When symptoms of threatened abortion are associated with a satisfactory pregnanediol output, it seems likely that the symptoms are not due to a deficiency of progesterone but to vitamin-E deficiency, which the patient's own progesterone is unable to counteract. In this event it appears rational to treat such cases with vitamin E rather than with progesterone.

Shute has repeatedly asserted that vitamin E in any dose is valueless in cases of habitual abortion, but he somewhat arbitrarily defines the latter as 'the condition in which at least the last three abortions have occurred consecutively at or before the third month' [Shute, 1940]. If this definition be accepted, only one (P97) and possibly two (P137) in the group of 'abortions at high levels of pregnanediol' belong to this category, so the remaining fifteen could suitably have been treated with vitamin E. However, it should be noted that three women, although belonging to Shute's category, were successfully treated with vitamin E only (P26, X, and P120 in Table XVI). The pregnanediol maxima in the vitamin E therapy group ranged from 46 to 90 mg./24 hr.—a satisfactory output.

Regarding the duration of therapy, MacGregor & Stewart [1939] express the opinion that progesterone need not be given beyond the 4th month, on the assumption that abortion in the early months is due to a deficiency of progesterone and is less likely to occur after the establishment of placental function. Reference to Table XV shows that abortion frequently occurred at or after the 4th month, and in four cases (P11, 47, 50, 97) the continuance of progesterone therapy failed to prevent abortion. Although the relation between pregnanediol output and progesterone secretion is not a strictly quantitative one, it would seem to be only a wise precaution to continue to administer progesterone when the values are persistently low, for fear of abortion or still-birth owing to foetal under-development. Shute actually recommends increasing doses of vitamin E, his argument being that placental development makes greater demands on the patient's store of vitamin E as pregnancy advances.

For purposes of comparison with the data of Currie [1939], Macdonald [1939] and MacGregor & Stewart [1939] our results have been expressed in a form similar to theirs in the Tables XVIII and XIX. Examination of these shows that, in the case of habitual abortion (Table XVIII), therapy whether by progesterone, or vitamin E or both, caused an improvement of 50–60 % on the number of previous successful pregnancies. The outstanding exceptions to these findings are 85 % improvement recorded by Macdonald [1939], 83 % by Iles [quoted by Currie, 1939], and 77 % by Vogt-Möller [1931, 1933, 1936][1] all for vitamin E. Macdonald's figures are all the more remarkable in that the number of previous successes was so

[1] Two, twenty and fifty-two cases respectively.

Table XVIII. *Habitual abortion: the effect of therapy—various authors' results compared*

Authors	No. of cases	No. of previous pregnancies	Previous pregnancies successes No.	%	After therapy successes No.	%
Vitamin E only						
Currie [1939]	81	274	47	17	62	76
Iles	8	29	5	17	8	100
Macdonald [1939]	18*	53	5	9·4	17	94
Macdonald [1939]	4†	6	0	0	2	50
Vogt-Möller [1931, 1933, 1936]	74*	?	0	0	57	77
Juhász-Schäffer [1933]	5	?	?	?	5	100
Watson [1936]	35*	97‡	8‡	8‡	25	71
Watson & Tew [1935, 1936]	11†	15	5	33	9	82
Hain	13	33	9	27	11	85
Progesterone + vitamin E						
Currie [1939]	8	?	?	?	7	87
Falls, Lackner & Krohn [1936]	30§	?	?	31‖	24	80
MacGregor & Stewart [1939]	20§*	65	10	15·4	14¶	64
Hain	23*	67	3	4·5	14**	61
	7†	13	6	46	6**	86

* Two or more spontaneous abortions.
† One spontaneous abortion.
‡ Data given for only twenty-eight cases.
§ Progesterone only.
‖ Figure for habitual and threatened abortion cases combined.
¶ In twenty-two pregnancies.
** Five breech presentations in the two groups combined.

Table XIX. *Threatened abortion: the effect of therapy—various authors' results compared*

Authors	No. of cases	No. of previous pregnancies	Previous pregnancies successes No.	%	After therapy successes No.	%
Vitamin E only						
Currie [1939]	40	?	?	?	36	90
Macdonald [1939]	20	30	21	70	8	40
Shute [1939]	59	?	?	?	?	68
Watson [1936]	15	20	13	65	11	73
Hain	5	7	3	43	4	80
No therapy						
Hain	24	15	12	80	6	25
Progesterone + vitamin E						
Hain	8	11	0	0	5	62·5
Falls *et al.* [1936]	28*	?	?	31†	24	86

* Progesterone only.
† Figure for habitual and threatened abortion cases combined.

low. To take into consideration the number of previous successful pregnancies probably affords a fairer basis for comparison than to compare percentage successes; for example, our own 85 % successes with vitamin E as against 61 % when progesterone ± vitamin E was given. The improvement was approximately 60 % by each method. The results do not support Shute's contention that vitamin E is useless in the treatment of habitual abortion.

The effect of vitamin E in cases of threatened abortion is not clear, as such varying results have been obtained by various workers. This is perhaps due to the fact that a common basis has not been employed. If the previous number of abortions affects the chances of success in a subsequent pregnancy, it is likely also to affect the chances when symptoms of threatened abortion are superimposed. It is important, therefore, to know the percentage of previous successes and failures. Furthermore, it is advisable to exclude all patients who did not receive therapy for more than 10 days.

In Table XIX Currie's successes are highly fayourable to vitamin E, whereas Macdonald's are not, especially when related to his previous successes. Shute's figure (68 %) comes between Currie's and Macdonald's, but a comparison with previous successes is not available. However, as compared with the absence of treatment there is a distinct improvement; this is more marked than in Macdonald's case (40 % as against 25 %). In our own small series, although the actual percentage of successes favours vitamin E therapy, progesterone + vitamin E yield a higher percentage improvement (62·5 % as against 37 %). As has already been stated, the object of the author's investigation was not a comparison of methods of therapy and consequently the data are neither clear-cut nor numerous.

Caesarian sections. The incidence of Caesarian section in the group of thirty-four women receiving progesterone + vitamin E therapy is high (11·7 %) (four cases); a fifth case occurs in the vitamin E group. In each of the four cases in the combined therapy group breech presentation occurred and was associated with a lax abdominal wall and flabby uterus; all the infants were born alive. A fifth case of breech presentation in this group (P88) resulted in foetal death in the process of manual delivery, as did also that in an occipito-posterior position. The possible part played by progesterone in causing immobility of the uterus leading to adoption of the breech position has been examined: in two instances (P31, P57) injections of progesterone at the rate of 10 mg. weekly were indeed continued as late as 4 and 6 days before operation, but in three others (P41, 64, 88) progesterone therapy ceased at 3½, 4 and 7 months, and two other cases of breech presentation (P142,[1] 29) were not so treated. It is, therefore,

unlikely that progesterone was in any way responsible for the high incidence of breech presentation in this series; at the same time consideration might be given to the possibility that the anomaly may be due to a physiological condition intimately related to a defective endocrine or vitamin metabolism. The series is a small one, but 11·7 % Caesarian sections in thirty-four cases compares unfavourably with the following percentages in American literature: 0·8 [Tamis & Klein, 1940]; 1·9 [Heard, 1940]; 2·96 [Mitchell, 1938], and 'at most 4 %' [Stander, 1933], figures, however, which relate to random series and not to a selection of more or less anomalous cases.

Sterility

Under this heading are considered women who had no viable offspring for at least the last five consecutive years (Table XX). There were only three cases of primary sterility, if by this is meant an entire absence of pregnancy, the three being P86, P111 and P141. Married in 1933, P86 had one ovary removed and the other ovary reduced to a mere fragment in 1936 and, with the aid of progesterone + vitamin E, carried through a pregnancy successfully in 1940–1. Following 'an overhauling for sterility a few years ago', P111 on similar therapy, had a live child to her first pregnancy in 11 years, whereas P141 after the same operative treatment but no endocrine or vitamin therapy, miscarried.

Particulars are also given in the same table of eight women married 5–19 years but without a single successful pregnancy. Six (75 %) of these had live children: two of them with the help of progesterone ± vitamin E therapy, two without either but only abundant rest, and two on vitamin E only. The honours are thus evenly distributed between the three forms of therapy. Of special note is P26, who had her first living child in 19 years after a series of early miscarriages.

The remaining six women in this table had had one or more live children followed by a lengthy interval during which there was either sterility or several miscarriages. Case P38 had a single live child in seven pregnancies in 11 years and this survived only 5 months. She had no therapy in any of these pregnancies nor did she rest; during the 1940 pregnancy, which terminated in a child now almost a year old, she had both progesterone and vitamin E and took a considerable amount of rest. Incidentally, it is noteworthy that, although in this case two injections of 5 mg. progesterone were administered weekly until as late as 6 days before parturition, labour was short and spontaneous. Two similar cases are described in the section devoted to Caesarian section (P31, P57).

In the other five women at the foot of Table XX five or more years of sterility followed the birth of two or more live children. In one case (P101) the facts suggest that the husband was at fault; repeated toxaemia was

obviously to blame in P68 who was sterilized on this account. The only successful pregnancies were those of P64, a diabetic aged 45, and P29, who at 42 and after 5 years' amenorrhoea, gave birth to twins.

Table XX. *Outcome of pregnancy in women sterile for upwards of 5 years*

se	No. of years married	Age	Obstetric history	Endocrine or vitamin* therapy	Outcome
6	8	27	No pregnancy	Progesterone and vitamin E	Live
11	11	34	No pregnancy	Progesterone and vitamin E	Live
41	5	—	No pregnancy	Rest in bed	Miscarriage: 3 months
3	9	36	2 still-born, 1 miscarriage at 2½ months	Progesterone	Miscarriage (toxaemia)
4	5	30	1 miscarriage at 3 months	Progesterone	Live
6	19	41	3 miscarriages at 2–3 months	Vitamin E	Live
1	11	34	2 abortions at 5–6 months	Progesterone and vitamin E	Live (Caesarian)
9	10	35	2 abortions 9 and 4½ years ago	Progesterone	Miscarriage
9	10	—	2 still-born (toxaemia)	Rest and diet	Live (toxaemia)
's X	7	32	4 miscarriages at 3–4 months	Vitamin E	Live
8	8	37	4 miscarriages at 6 and 7 months	Rest in bed	Live
8	11	29	6 still-born, 1 miscarriage at 3 months, 1 full term lived 5 months (1936)	Progesterone and vitamin E	Live
4	6 years sterile	45	3 full term (alive) before 1932; 1 still-born 1933 (toxaemia); sterility 1934–40; diabetic	Progesterone for 1 week only	Live
8	24	42	5 miscarriages after 9 live children (all 14 toxaemias)	None	Pregnancy interrupted; patient sterilized
101	11 (to 2nd husband)	—	3 live full term to 1st husband; 8 or 9 miscarriages to 2nd husband	Progesterone and vitamin E	Miscarriage
135	?	35	5 miscarriages after 8 live children	None	Miscarriage
29	?	42	2 live; no menstruation since birth of 2nd child 5 years ago	None	Live twins at 8 months (2nd one a breech)

* For amounts see Tables XV and XVI.

Examination of the records of the women listed in Table XX shows that only in those who miscarried was the pregnanediol output low; in all the others the maxima reached were between 40 and 60 mg./24 hr., which are satisfactory figures. In two cases values above the normal were recorded: in P64 a diabetic with toxic symptoms (137 mg.) and in P135 whose output at 4 months was 27 mg./24 hr.; the latter aborted 12 days later. It is impossible to assess the effect of progesterone and vitamin E therapy in maintaining normal pregnanediol values. No indication is given in this series of the part undoubtedly played by rest, as particulars are given in an earlier table.

Interesting cases

Certain interesting cases are worthy of a brief record; prolonged amenorrhoea, generally associated with other symptoms, caused pregnancy to be suspected.

Case P39 (aged 40) had not menstruated since a hydatidiform mole expelled Nov. 1939. On 8/3/40 A.Z. weakly positive; 19/3/40 A.Z. definitely positive; 31/3/40 no pregnanediol. The patient was not pregnant; one is unable to explain a positive Aschheim-Zondek.

Case P29 (aged 42) had not menstruated since birth of second child 5 years ago when persistent post-partum haemorrhage for 4 weeks. On 7/3/40 uterus $=4\frac{1}{2}$ months' pregnancy; 12/3/40 A.Z. positive; 17/3/40 pregnanediol output 40 mg. Ante-partum haemorrhage in April and again in May for which the patient was in bed for 4 weeks. Live twins born July (8 months) of which the second was a breech presentation. Only one slight menstrual period occurred in ensuing 7 months.

Case P63 (aged 47): amenorrhoea 5 months; abdominal swelling, morning sickness, had not been pregnant before, foetal movements absent. A.Z. negative; pregnanediol 1–2 mg./24 hr. Not pregnant, but N.B. some pregnanediol although no menstruation.

Case P32 (aged 41); 6 para; $5\frac{1}{2}$ months' amenorrhoea; uterus $=5\frac{1}{2}$ months' pregnancy. At $6\frac{1}{2}$ months A.Z. negative; at $6\frac{3}{4}$ months pregnanediol output 3·0 mg./24 hr. Similar case to P63.

Case P71 (aged 40); uterus = 8 months at 3 months; foetal movements absent; X-ray negative for pregnancy; severe hydramnios. At 5 months A.Z. positive and pregnanediol output 54 mg./24 hr. at 6 months; anencephalic monster removed at $6\frac{1}{2}$ months.

Case P73 (aged 33); 6 months' amenorrhoea; no foetal heart or parts. A.Z. positive at $6\frac{1}{4}$ months; pregnanediol 65 mg./24 hr. at $6\frac{1}{2}$ months; normal pregnancy.

Case P72 (aged 26); 7 months' amenorrhoea; uterus slightly enlarged but not $=6\frac{1}{2}$ months. A.Z. negative; pregnanediol nil.

Case P162 (aged 27); 7 months'amenorrhoea; uterus much enlarged; symptoms of threatened abortion. Pregnanediol nil; A.Z. negative. Curettage done immediately.

Case P46 (aged 44); 6 para; uterus = 7 months at 5 months but no foetal heart or movements felt. A.Z. positive at 5 months and pregnanediol 21 mg./24 hr. Pregnancy + toxaemia.

Case P75; uterus = 5 months at 3 months; severe haemorrhage for some weeks; suspected hydatidiform mole. Quantitative A.Z. positive at $2\frac{1}{2}$ months; pregnanediol 5·5 mg./24 hr.

Case P49; rapidly increasing weight; gained 1 stone in $2\frac{1}{2}$ months; $6\frac{1}{2}$ months' amenorrhoea. At 7 months, pregnanediol 41 mg./24 hr. Twins.

Case P81; three negative A.Z.'s had been returned by a London laboratory; pregnanediol output was 22 mg./24 hr., showing patient was pregnant.

The above series demonstrates the help that a knowledge of the pregnanediol output can afford in differential diagnosis. A negative Aschheim-Zondek test after 6–7 months' amenorrhoea is not informative owing to the lowering in gonadotrophin excretion which occurs normally at 6–7 months' pregnancy. The excretion of considerable amounts of pregnanediol

in such circumstances is a clear indication of pregnancy, although not indicative of a living foetus (as can be seen from the section devoted to *Foetal Death*, p. 43).

Three of the above merit special comment. In case P 162, after the negative pregnanediol finding, an Aschheim-Zondek test was done before it was thought safe for a dilatation and curettage to be performed. This is always advisable when the symptoms have made it clear that pregnancy existed up to this point. The low pregnanediol output in a case of hydatidiform mole (P 75) was to be expected but had no diagnostic value, such as was provided by the Aschheim-Zondek finding. From cases P 32 and P 63 it is clear that small amounts of pregnanediol may be excreted after the menopause. Indeed, P 32 was excreting as much as some women at the peak of a cycle which terminates in menstruation and one is inclined to speculate why menstruation occurs in one case and not in the other.

Hydramnios in four women was associated with high values of pregnanediol.

Hyperemesis gravidarum: in three out of four women with hyperemesis the pregnanediol output was normal at the time of the symptoms; in the fourth the ovum was dead and consequently only a trace of pregnanediol was recoverable. Aschheim-Zondek tests gave a standard positive in all cases, and the excretion of combined oestrone ascertained in a single case was normal for the stage of pregnancy. Thus no hormone abnormality was found associated with the condition; this is in keeping with the findings of Browne *et al.* [1938].

Transverse myelitis occurred in a woman of 24 when 6 months pregnant (P 113); the condition rapidly developed into complete paresis of the lower limbs and labour had to be induced at 7 months, when a foetus 3–4 weeks macerated was delivered. The pregnanediol values associated with the condition were low (12 mg. at 4 months, 17 mg. at 5 months and 28 mg. at 6 months at which point the foetus died). The low pregnanediol output taken in conjunction with the findings of Einarson & Ringsted [1938] and of Bicknell [1940] that vitamin E has a specific action on certain portions of the spinal cord and its nerve roots, as well as on the skeletal musculature, make one speculate as to the effect that its early administration might have had in this case. The condition cleared up on the termination of pregnancy.

The incidence of congenital anomalies. The wisdom of preventing spontaneous abortion is considered by Shute [1939] in a review of the literature: congenital anomalies were found to occur in 5·6 % of viable children born to mothers treated with vitamin E or progesterone for threatened abortion. In our series, consisting of approximately 150 cases of threatened or habitual abortion, sterility and toxaemia, only six instances of foetal

abnormality are recorded (4 %). Progesterone and vitamin E therapy were given to only three of the mothers involved, for periods exceeding 10 days. Hydramnios occurred with four of the infants, one of which was anencephalic. Three of their mothers were aged 40–45, and one of these was diabetic; the fourth, aged 28, had had three miscarriages. The remaining two mothers received both progesterone and vitamin E throughout pregnancy on account of sterility; their ages were 44 and 36; one aborted a monster having four legs, and the other was delivered at term of a live male having spinal curvature, who survived only 6 weeks.

DISCUSSION

In the foregoing study certain facts have been elucidated regarding abortion and threatened abortion which, besides throwing new light on the two conditions themselves, may have a bearing on the problem of parturition. Enumerated they are as follows:

(1) In over 60 % of patients in whom symptoms of threatened abortion were present the outputs of gonadotrophin and pregnanediol were such as characterize normal pregnancy and were not diminished.

(2) Abortion occurred in seventeen cases within 1–16 days of a satisfactory pregnanediol output, and in others within a similar interval after a positive Aschheim-Zondek reaction.

(3) On the other hand, (*a*) in spite of very low pregnanediol values (9–18 mg.), foetuses aborted at 6 and 7 months were alive and of normal development for the stage of pregnancy; and (*b*) in several cases, pregnancy was maintained and abortion did not occur until either very little or no pregnanediol was excreted.

(4) Moreover, pregnancy can proceed normally in the temporary absence of any pregnanediol output in its early stages.

(5) Further, (*a*) amounts of injected progesterone which should have been adequate to maintain pregnancy failed to prevent abortion, and (*b*) progesterone (10 mg. weekly) given over a considerable period and to within 4–6 days of parturition, did not prevent a spontaneous and even short labour; (*c*) up to the onset of labour, pregnanediol values such as the following were excreted per 24 hr.: 22 mg. (P7), 34 mg. (P8), 55 mg. (Case 70), 28 mg. (P40), all of which were amounts justifying a continuance of the pregnant condition. One would expect a drop to negligible values if parturition is due to a cessation in the production of luteal hormone, as has been claimed.

In the absence of knowledge of the factors which determine the concentration of hormones in a given fluid or of the processes controlling the elimination of hormones, it is necessary to take for granted that some relation exists between the amount of progesterone secreted and the

amount of sodium pregnanediol glucuronide excreted. On account of the failure of Hamblen, Ashley & Baptist [1939] and of Stover & Pratt [1939] to recover any pregnanediol after injecting progesterone, added to his own inability to recover more than traces of the glucuronide, Cope [1940*c*] expresses the opinion that the assumption that pregnanediol excretion bears any quantitative relation to corpus luteum activity is at present unsupported by evidence. Venning & Browne [1937*a*, 1938], however, recorded recoveries ranging from 12 to 46 % of the amount injected, and have recently demonstrated that recovery depends on the normality of the various mechanisms involved in the excretion of the glucuronide; the problem is fully discussed and exemplified by them [Venning & Browne, 1940].

Moreover, the production of a premenstrual endometrium by Kaufmann [1935] with some 35 mg. progesterone given over 7 days, together with the calculation of Corner [1937] that something of the order of 5 mg. progesterone per day is produced during the premenstrual period, carry added significance when recoveries of as much as 20–60 mg. pregnanediol were recorded soon afterwards for the luteal phase by Venning & Browne [1937*a*, 1937*b*], and later by Hain & Robertson [1939] as well as by others. We have accordingly, in the brief discussion which follows, assumed that a high and low pregnanediol output are not entirely without significance or incapable of being related to the conditions with which they are associated.

The facts that have been enumerated above make it clear that (*a*) abortion and parturition occur in spite of a plentiful progesterone secretion supplemented even by the injection of considerable amounts of progesterone; (*b*) on the other hand, pregnancy and foetal development can proceed normally in spite of a very low pregnanediol output. In a number of cases such pregnancies terminate suddenly and prematurely, but up to that point foetal development has not been impaired, a fact which suggests that a low progesterone secretion is capable of maintaining pregnancy, and one which the data of the previous paragraph belie.

The difficulty lies in trying to reconcile these conflicting findings. Abortion and the symptoms of threatened abortion would appear to be unrelated to progesterone secretion in that adequate amounts of the latter are unable to prevent either condition. The logical conclusion is that a factor making for abortion ('abortive factor') exists over which progesterone has little or no power; when this factor is inactive, pregnancy is capable of being maintained by even small amounts of progesterone.

In view of the antagonism known to exist between oestrogen and progesterone, the possibility of oestrogen being the factor in question should be considered:

Is oestrogen the abortive factor?

(*a*) Abortion both in human beings and in animals may occur as the result of vitamin E deficiency, which Shute [1936] has shown to be associated with a high blood oestrogen; vitamin E is anti-oestrogenic; progesterone cannot override vitamin E deficiency. Patients habitually aborting require more vitamin E than the normal pregnant woman [Vogt-Möller, 1936].

(*b*) Some days before the onset of labour there is a marked increase in the concentration of combined oestrogen in the urine and this phenomenon may be a necessary prelude to the labour process, acting either directly or via the pituitary or both.

(*c*) Emotional influence may act upon the pituitary in such a way as to bring about so marked an increase in the excretion of oestrogen as to result in abortion. This indicates that nervous stimuli can indirectly modify the concentration of hormones in body fluids; the mode by which this is brought about has been indicated by the recent work of Vazquez-Lopez [1940–1] in the horse.

(*d*) As has been suggested by Marrian [quoted by Robson, 1940*b*, p. 211] enzymes may, under favourable conditions, release the active hormone from the conjugated compound and thus lead to a concentration of free hormone in an organ (for example, the uterus) higher than that present in the blood. The author has been unable to find an association between the concentration of free oestrogen in the urine and the onset of labour, but this does not preclude some such possibility as that suggested by Marrian in this paragraph.

Venning & Browne [1940] and Pattee, Venning & Browne [1940] have recently observed that the injection of oestrogen over a short period causes a decrease in the total output of pregnanediol, and a shortening of the luteal phase of the menstrual cycle. On this analogy one would anticipate that, if oestrogen were the abortive factor in those cases in which abortion occurred at high levels of pregnanediol excretion, the output of pregnanediol would have been low. It is possible that in the pregnant woman, in whom much larger amounts of progesterone and oestrogen are secreted than in the non-pregnant woman, the added oestrogen might cause local disturbances (for example, in the uterus) more readily than it would affect the secretion and excretion of progesterone. It is clear from our own two cases that a high oestrogen level (endogenous or injected) can be associated with, or indirectly cause, abortive symptoms without reducing the pregnanediol output. The cases in question are: P108, a cardiac patient, in whom a therapeutic abortion was tried with the aid of a powerful oestrogen plus other drugs, and who aborted within a few hours of a high pregnane-

diol excretion; and P8 who, under emotional influence, experienced a sudden excessive excretion of oestrogen as the result of which she nearly aborted: the pregnanediol output was unaffected.

Is the abortive factor concerned with the control of the concentration of progesterone in the blood?

Since the concentration of sex hormones in the blood is obviously of paramount importance, variations in the factor controlling such concentration can have far-reaching effects on pregnancy. It is clear that the process of hormone elimination is closely linked up with such control and it follows that the elimination of pregnanediol might be such as to bring about a lowering of its concentration (and of that of progesterone) in the blood, resulting in abortion.

Is the abortive factor that which controls the pre-parturition rhythm?

Further evidence has been supplied of the existence of rhythmic fluctuations in hormone excretion during the last stages of pregnancy; the rhythm is such as to suggest a definite extraneous control. Instances are given in this study of three full-term labours in which the phenomenon was manifest, and less complete data in a case of spontaneous abortion (case P11) suggest that the same factor may have been at work. There is no evidence to show whether this was the pituitary or some other controlling agent.

Is abortion due to progesterone-deficiency?

That abortion in the presence of a high pregnanediol output (and, presumably, of an adequate secretion of progesterone) may be caused by an unspecified abortive factor is perhaps a rather obvious conclusion. It does not allow, however, for another aspect of the matter which concerns the efficient utilization of the hormone before excretion. Its excretion in considerable amounts does not necessarily imply either adequate utilization or normal endometrial development and placental function, and an abortion which occurs in spite of a satisfactory pregnanediol output may still be a progesterone-deficiency abortion, such as occurs when the amount of progesterone secreted is unequal to the growing demands of the foetus. Such abortions are generally (but not only) encountered in the early stages of pregnancy and are associated with low pregnanediol values.

Several instances of abortion in spite of high pregnanediol excretion figures have been cited in the literature: Cope [1940*a*] mentions three, Browne *et al.* [1939] one, and Randall & Wilson [1939] a case of threatened premature labour in which high pregnanediol values were associated with a high gonadotrophin and low oestrin output. The interesting feature in

common is that in no case was the progesterone secreted capable of preventing the abortive symptoms. From these and our own group of seventeen cases, it is clear that the following statement by Robson [1940*b*, p. 251], voicing the theory commonly held, needs modification: 'In cases of habitual abortion in which abortion regularly takes place in the early stages of pregnancy, this is preceded by a gradual fall in the amount of pregnanediol excreted in the urine and thus presumably by a decrease in the production of progesterone.'

Although it must be borne in mind that the mechanisms of abortion and parturition may be two different things, yet the bearing that these facts have on the causation of parturition is three-fold:

(1) A high oestrogen level is of itself insufficient to set in train the events which precipitate labour, but may play a secondary part in the process.

(2) The values of pregnanediol and oestrogen excreted at the onset of labour (and abortion) are unimportant and may be high or low; there exists, apparently, a factor concerned with the control of their elimination and concentration in the body and it is this factor which is probably directly responsible for parturition. This control is cyclic and rhythmic in its effect.

(3) There is little doubt that the combined secretion of oestrogen and progesterone is essential for the maintenance of pregnancy, but their utilization may vary from individual to individual and at different stages of pregnancy; the peak in the output of both hormones which occurs during the last month of gestation may denote increased secretion or increased elimination[1] or a change in utilization. It may, on the other hand, indicate that the pituitary responds differently to nervous stimulation at different stages of pregnancy, or that there is a change in the nature of its nervous control associated with parturition.

The time seems to have come when it is neither sufficient nor entirely true to say that parturition is due to a fall in the levels of oestrogen and progesterone and efforts might profitably be concentrated on controlling their secretion and excretion. In this way much might be learnt not only of the cause of parturition but also of the maintenance of pregnancy and the prevention of abortion.

The success attending progesterone or vitamin E therapy in cases of habitual and threatened abortion is, without doubt, largely dependent on the cause or causes underlying the condition. In the absence of any means for determining whether this is faulty utilization of progesterone, a deficiency of the hormone, vitamin E deficiency or an unspecified abortive factor, it would seem to be safest to combine progesterone and vitamin E therapy, of which the latter seems as successful as the former. Clinicians

[1] This aspect was more fully discussed by Hain [1940].

should be cautioned against interpreting a satisfactory pregnanediol output as an infallible indication of a satisfactory pregnancy, owing to the number of abortions taking place at such values, but be guided by the past obstetric history of the patient. The same applies to positive Aschheim-Zondek and Friedman findings.

SUMMARY

1. The pregnanediol and oestrogen excretion was ascertained at frequent intervals throughout pregnancy in four women receiving therapy for recurrent abortion and in a normal woman during the last 3 weeks of pregnancy; one aborted at the 22nd week. Neither labour nor abortion were associated with a rise in the excretion of free oestrogen. Rhythmic fluctuations in hormone output, suggesting an extraneous control, occurred at the approach of term in the three full-term pregnancies and may have been present in the patient that aborted. There was no clear evidence of cyclic variations in hormone output at monthly intervals. Suggestions are made as to the possible cause of previous abortions in the cases investigated.

2. The gonadotrophin and pregnanediol outputs associated with symptoms of threatened abortion are examined singly and together. In over 60 % of cases the excretion of both hormones was such as occurs in normal pregnancy.

3. Abortion occurs both at high and at low levels of pregnanediol excretion; seventeen cases of the former are described.

4. Pregnanediol outputs were ascertained throughout pregnancy in about 100 cases which terminated successfully; their value as a guide to therapy and prognosis and as throwing light on the nature of normal function is examined. Attention is drawn to the occasional absence of pregnanediol excretion in early pregnancy.

5. The pregnanediol excretion in cases of toxaemia, diabetes and foetal death was also ascertained.

6. The effects of progesterone therapy, vitamin E and rest in cases of threatened and habitual abortion were analysed and compared with other authors' results

7. The pregnanediol output is given throughout pregnancy in a number of women with over 5 years' sterility.

8. Certain cases are cited as demonstrating the aid to clinical diagnosis afforded by a knowledge of the pregnanediol excretion.

9. The discussion deals chiefly with the possible causes of abortion, and the mechanism of parturition.

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THE ENDOCRINE SYSTEM AND HAIR GROWTH IN THE RAT

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THE literature dealing with the influence of endocrine organs on hair growth is not extensive, and the results reported are by no means concordant. In the rat there seems to be a specific influence of the thyroid gland, the administration of which accelerates hair growth in some normal animals, but not all, whereas underfed animals respond more uniformly [Chang, 1926; Chang & Feng, 1929]. Chang & Feng [1929] also noted that thyroidectomy slows the rate at which new hair grows, and found no difference in response between the sexes. They measured the time elapsing before growth on a shaved patch appeared to be complete. Butcher [1940] found that thyroxin and various irritants such as xylene or capsicum decreased the time elapsing before the first appearance of new hair in underfed albino rats, whereas well-fed animals were not much affected.

Freud [1934] found that normal, thyroidectomized, epiphysectomized and castrated male rats take 6–11 days to show a uniform new coat of hair over the whole of a denuded (plucked) area, but that hypophysectomized rats take 14–21 days. The period was restored to normal in hypophysectomized rats injected with a 'non-purified growth hormone'. Snow & Whitehead [1935] also concluded that hypophysectomy decreased the rate of regeneration of hair after shaving in the albino rat, and that the injection of extracts of fresh anterior lobes of sheep or cattle restored the growth rate to normal.

Butcher [1939] showed that the hair growth of adrenalectomized albino rats is greater than normal, and Butcher & Richards [1939] further record that adrenalectomy increases the hair growth of underfed rats, in which the adrenals normally hypertrophy [Jackson, 1915], and in which hair growth is retarded [Butcher, 1937]. The adrenal glands of rats thus appear to be capable of inhibiting or slowing the growth of hair, a finding which does not accord with the occurrence of hirsutism, apparently attributable to the adrenal glands, which may occur in human subjects. Stein & Wertheimer [1941], however, have shown that adrenalectomy causes a transient increase in the rate of hair loss in rats, which occurred as markedly in medullectomized animals as in those from which the adrenals had been completely removed. This hair loss was inhibited by acid extracts of

adrenal medulla, and by synthetic *l*-adrenalin. The authors conclude that the hair moult in rats is regulated by the adrenal glands.

The above account covers most of the positive findings for the rat. In other species, various actions of different endocrine glands have been suggested or recorded. Hensel [1938] finds that hair growth in the guinea-pig is retarded by progesterone, but is not affected by oestrogens or by pituitary extracts. Hu & Frazier [1940] found that an oestrogenic extract of human pregnancy urine accelerated the first appearance of new hair on shaved patches in rabbits but did not increase its subsequent rate of growth; in fact, the oestrogen-treated buck showed a slowing of the growth rate of the hair. The restoration of hair in man after spontaneous baldness appears to follow treatment with extracts of anterior pituitary glands in many cases [Bengston, 1931, 1934; Kohn, 1932], but Lord [1933] did not obtain good results with such therapy. The implantation of sheep pituitary glands has also been reported to cure some cases of alopecia [Stanley & Hawkins, 1933; Kylin & Dicker, 1939; Kylin, 1940]. Tscherne [1939] finds that hair growth is increased by the treatment of castrated and post-menopausal women with oestrogens. Reviews of the literature on human cases in which various types of endocrine therapy have been tried are given by Stein [1936], Mayr [1939] and Tscherne [1939].

The authors cited above have used various methods for measuring the hair growth occurring in their experiments, ranging in refinement from straightforward observations of the time taken for the new coat to start growing, or to appear fully grown, to assessments of the weight of hair produced over the denuded area. Some investigators have also measured the rate at which the growing hair increased in length. In a mammal such as the rat, more than one type of hair occurs, and the rate of growth may vary over different parts of the body. Care must therefore be taken in comparative work to measure the same type of hair in control and test groups, and to denude corresponding areas on the animals compared. The meaning of the term 'hair growth' must also be defined, as a particular treatment may (*a*) initiate an unusually rapid onset of growth on a denuded area, (*b*) cause more follicles to produce hair than would normally be the case, (*c*) cause the hair to increase more rapidly in length than is normal, (*d*) increase the weight (diameter ?) of individual growing hairs, with or without effect (*c*), or (*e*) may have corresponding retarding effects.

MATERIAL AND METHODS

Details of the strains and body weights of rats used will be given at appropriate places in the text. The area studied in all instances was the flank, a portion of which, measuring about $5 \times 2{\cdot}5$ cm., was shaved or clipped, or depilated with barium sulphide paste. Samples of the growing

hair were plucked at frequent intervals during the earlier tests, and the length of the four or five longest hairs measured with a pair of dividers and the average figure taken—variation often being very small. Care was taken to sample the area evenly, and the fine undercoat hairs were excluded. A record of the extent of regeneration was also made on each occasion, since very few rats grow a new coat over the whole of the denuded site.

The first criterion of growth was the length of the hair. The second criterion was the area of regeneration, and it was found that, for statistical purposes, the proportion of rats in a group whose shaved areas were completely covered by a coat of new hair at any given time afforded a good index of the extent of regeneration, and avoided attempts at complicated planimetric measurements.

In some later tests, after data relating to the normal course of regeneration of hair had been established, fewer measurements were taken, at longer intervals, although observations were usually extended for a sufficient time to observe the incidence of the restoration of a full coat over the denuded region in almost all animals, or until it was apparent that a particular group of rats was significantly different in its response from the controls.

CONTROL AND EXPERIMENTAL DATA

Hair growth in untreated albino rats

Preliminary tests were made with forty albino rats from the Glaxo Laboratories' Wistar colony, twenty of each sex, ranging in body weight from 114 to 157 g. The experiment was set up so as to determine the course of regeneration of hair in each sex after depilation by shaving or with barium sulphide paste, to compare the effect of denuding both flanks as against one flank, and to compare left and right sides. The results up to 22 days after the beginning of the test are shown in Table I. The twenty rats of each sex were divided into four groups; the first was shaved on the left side, the second shaved on the left side and depilated with paste on the right side, the third depilated with paste on the left side, and the fourth depilated with paste on the left side and shaved on the right side. Measurements were taken at 5, 8, 11, 14, 18 and 22 days from the start of the test, after which the coat in the most advanced animals was substantially full, but observations of the completeness of regeneration in others were continued until the 42nd day.

The way in which hair grows over a depilated area is peculiar and does not seem to have been noted by other investigators, whose reports usually convey the impression that a new, complete coat springs up over the whole of a shaved patch, and then grows to normal length. In all of the tests made with rats, with very few exceptions and those only in young animals

Table I. *Hair growth in the albino rat after depilation, five rats per group*

Group	Sex	Av. body wt. g.	Method of depilation		Average hair growth in mm. on day											
					5		8		11		14		18		22	
			Left side	Right side	Left side	Right side	Left side	Right side	Left side	Right side	Left side	Right side	Left side	Right side	Left side	Right side
1	♂	141	Shaved	—	0·0	—	0·0	—	10·8	—	12·0	—	16·8	—	18·5	—
2	♂	139	Shaved	Pasto	7·6	7·4	9·9	8·7	12·7	10·0	15·4	11·1	18·3	14·3	21·2	16·3
3	♂	144	Pasto	—	7·8	—	10·2	—	13·2	—	15·0	—	19·4	—	22·5	—
4	♂	136	Pasto	Shaved	7·5	7·5	10·5	9·9	14·3	13·4	15·2	15·4	20·4	20·6	22·6	22·7
5	♀	127	Shaved	—	4·4	—	7·5	—	9·4	—	11·3	—	15·2	—	17·2	—
6	♀	127	Shaved	Pasto	5·9	5·8	8·1	8·7	10·0	11·0	10·9	12·4	14·5	15·8	17·0	17·2
7	♀	121	Pasto	—	4·7	—	6·8	—	8·9	—	11·0	—	14·4	—	17·6	—
8	♀	129	Pasto	Shaved	3·0	4·1	6·9	7·0	9·3	9·3	11·0	9·9	14·2	11·8	17·4	13·5
1–4	♂	140	Mean (left side) $\pm \sigma_m$		7·45 ± 0·271		9·90 ± 0·252		12·75 ± 0·566		14·40 ± 0·705		18·73 ± 0·547		21·20 ± 0·646	
1–4	♂	140	% full coats		0·0 ± 0·0		0·0 ± 0·0		5·0 ± 4·9		80·0 ± 8·9		95·0 ± 4·9		95·0 ± 4·9	
5–8	♀	126	Mean (left side) $\pm \sigma_m$		4·50 ± 0·044		7·33 ± 0·071		9·40 ± 0·058		11·05 ± 1·131		14·57 ± 0·054		17·30 ± 0·754	
5–8	♀	126	% full coats		0·0 ± 0·0		0·0 ± 0·0		0·0 ± 0·0		55·0 ± 11·1		90·0 ± 6·7		95·0 ± 4·9	

(see later), both males and females failed to grow new hair over the whole of the denuded area immediately. Instead, a patch or patches of hair commenced growing straight away, but other areas remained dormant. The hairs measured in this work have been taken from these early-growing patches. Between 11 and 18 days after the beginning of the test, the remaining dormant areas then produced a later crop of new hair, which, with few exceptions, covered the rest of the bared patch, and the rat was then said to have a 'full coat', in the sense that no bare region remained. The later crop of hair grows at the same rate as did the first, and eventually catches up with it. A glance at Table I shows that the percentage of full coats in both males and females rises steeply from 0–5 on the 11th day to 90–95 on the 18th day, and in fact the majority of animals start the later hair growth practically simultaneously on the 13th–15th days.

Female rats sometimes fail to grow any hair immediately, and the onset of all growth may be delayed until the period of the 2nd growth phase. A few males also show the same phenomenon, but in the present (control) series no males, but eight females failed to show immediate growth of hair. This has caused the variance of the data for the female growth curve to be consistently greater than that for the males, particularly in the early stages, and if such 'non-starters' are omitted from the calculations the rate of female hair growth is almost as high as for males, but such an arbitrary procedure has not been followed, as the inclusion of the non-starters permits expression, in the growth curves, of a significant sex difference, without seriously impairing the sensitivity of tests at the later stages of hair growth.

The relative effects of shaving and of depilating with paste on one or both sides, and the sex difference and difference in response between right and left sides were examined by considering the data for the 11th day, about half-way through the growth period (Table II). The whole of the

Table II. *Analysis of variance of hair growth on the 11th day for the rats listed in Table I*

Variance	Sum of squares	Degrees of freedom	Mean square
Between sexes	102·238	1	102·238
Between treatments	0·5078	1	0·5078
Between sides	10·30	2	5·150
Error	856·167	55	15·567
Total	969·213	59	—

data for the complete test might have been used for these comparisons, but in view of the straight-line relationship between growth and time (see below) it did not seem likely that the extra calculations involved would give any much greater accuracy.

It will be seen that the symmetry of the test is such that the effects of shaving and of depilating with paste may be compared from the corresponding totals for each treatment. The effects of denuding both sides or one only, together with any differences due to the actual side of the body denuded, may be compared from the three totals under those headings, the 2 side : 1 side comparison being made from left sides only, and the sex difference may be similarly estimated. The mean growth at 11 days on shaved areas (both sexes together) was 10·93 mm., and on areas depilated with paste was 11·12 mm. The growth on the left sides of animals with undenuded right sides was 10·58 mm., on the left sides of animals with depilated right sides (shaved + paste) was 11·58 mm., and on the right sides was 10·93 mm. The hair of males grew 12·40 mm., on the average, and that of females grew 9·65 mm.

Effects other than a sex difference in growth thus appear to be negligible, and this is confirmed by an analysis of variance (Table II). The only significant difference in variance from the error term is for that between sexes ($P < 0{\cdot}02$, where P is the probability of as large a difference occurring by chance). It thus appears that hair growth is not easily affected by minor factors such as the method of depilation or the total amount of hair removed. Hair growth was also uncorrelated with body weight in the small range investigated in this series.

The same rats were next used for a further test, in which those which had previously been depilated on the left side only were now shaved on both sides, depilation by barium sulphide being abandoned (Table III). No significant differences were found in this second growth test, except that due to sex, which was approximately the same as before. The data in Tables I and III were therefore combined for making a growth curve, shown in Fig. 1, from which it is seen that the growth of hair in both sexes bears a linear relationship to time, from shortly after its inception to near the length of the normal coat. Regression lines fitted to the data are $y = 0{\cdot}8716t + 2{\cdot}634$ for males, and $y = 0{\cdot}7485t + 0{\cdot}988$ for females, where y = hair growth in mm. on day t. Males thus grow a little under 1 mm. of hair per day, and females about $\frac{3}{4}$ mm.

The standard errors given in Tables I and III are the standard errors of the means. They were calculated from the left side measurements only in the first series (Table I), in order to avoid weighting the means by the inclusion of two readings from some animals and one from others. In the second series of measurements (Table III) they were calculated from all of the measurements, as each animal was shaved on both sides. From the data for the two series combined, female rats show no significant correlation between growth on the two sides of the body ($r = 0{\cdot}252$, $P > 0{\cdot}1$), but males show a highly significant correlation ($r = 0{\cdot}810$, $P < 0{\cdot}001$). This

Table III. *Hair growth in the albino rats listed in Table I after a second depilation, five rats per group, all over 150 g. body weight*

Group	Sex	Previous treatment: Left side	Previous treatment: Right side	Average hair growth in mm. on day 4: Left side	4: Right side	8: Left side	8: Right side	12: Left side	12: Right side	16: Left side	16: Right side
1	♂	Shaved	—	4·8	4·5	8·6	8·1	12·6	9·7	17·4	15·1
3	♂	Depilated with paste	—	5·1	6·3	10·6	10·3	15·9	15·1	18·3	16·6
5	♀	Shaved	—	4·1	4·4	6·4	7·3	11·2	9·7	14·3	13·7
7	♀	Depilated with paste	—	3·1	4·7	5·2	7·4	8·1	11·7	11 4	13·4
1+3	♂	See above	—	4·95	5·40	9·60	9·20	14·25	12·40	17·85	15·85
5+7	♀	See above	—	3·60	4·55	5·80	7·35	9·65	10·70	12·85	13·55
1+3	♂		Mean $\pm \sigma_m$	5·18 ± 0·531		9·40 ± 0·821		13·33 ± 1·061		16·85 ± 0·842	
1+3	♂		Full coats %	0·0 ± 0·0		0·0 ± 0·0		0·0 ± 0·0		95·0 ± 4·9	
5+7	♀		Mean $\pm \sigma_m$	4·08 ± 0·552		6·58 ± 0·932		10·18 ± 1·230		13·20 ± 1·064	
5+7	♀		Full coats %	0·0 ± 0·0		0·0 ± 0·0		0·0 ± 0·0		85·0 ± 8·0	

difference is in part due to the moderately frequent failure to grow any hair on one side in females, while the other side produces a growing patch.

The two series also agree as regards the growth of full coats, the second showing as sharp a rise, from 0 to 85%, within 4 days. In order to make sure that the areas which remained dormant were not damaged by depilation, a group of nine male rats was carefully clipped with scissors so as to leave a very short even stubble over the area. These rats grew hair

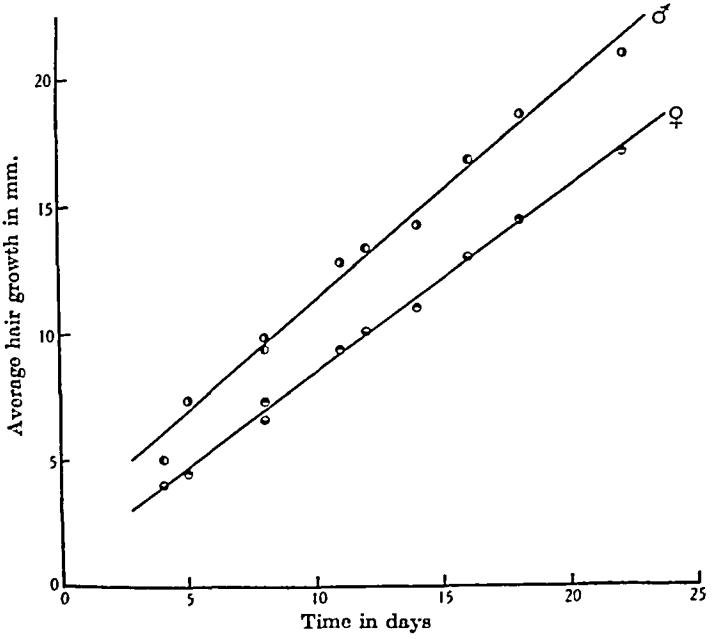


FIG. 1. Hair growth in albino rats. ◑ Males depilated for the first time. ◐ Males depilated for the second time. ◓ Females depilated for the first time. ◒ Females depilated for the second time.

at the same rate as those above, but were about 1 mm. in advance throughout, and showed exactly the same phenomenon as regards the incidence of temporarily dormant areas. On the 8th day after clipping, no rat showed a full coat, i.e. the stubble on part of the clipped area in each rat had not grown; by the 13th day, one was fully growing; and by the 17th day, seven of the nine rats had full coats of growing hair. The second phase of hair growth shown by shaven or depilated rats is therefore not related to injury to the follicles.

Hair growth in untreated coloured rats

In order to investigate the effect of coat colour on hair growth, dealers' rats, which were throwing albinos, fawns, browns and hooded (brown and white) rats, intermixed in litters, were used. The requisite number of males

of each type was obtained from as few litters as possible, in order to reduce inter-litter differences, but it was not possible to arrange a test in which litters were distributed among colour groups in a balanced manner, nor to obtain rats as uniform in body weight as I should have liked. The rats were first shaved when young, at body weights of from 37 to 103 g., and in a second test they were used at 119–200 g. The rats of the second test were mainly those which had already been used when younger.

Table IV. *Effect of coat colour on hair growth in young male rats*

Group	No. of rats	Colour	Average body weight g.	Hair growth in mm. on day 3	7	10	13	16	19
1	5	Albino	65	0·8	1·8	3·5	5·5	9·1	11·1
2	4	Fawn	56	2·1	4·2	6·4	8·6	10·9	14·5
3	4	Brown and white	68	3·5	7·0	9·3	12·4	13·0	15·4
4	5	Brown	78	3·3	7·6	9·9	11·4	13·4	15·1
				Number with full coats					
1	5	Albino	65	0	1	1	1	2	3
2	4	Fawn	56	0	1	1	1	2	2
3	4	Brown and white	68	1	2	2	3	3	3
4	5	Brown	78	2	2	3	3	3	4

Table V. *Effect of coat colour on hair growth in adult male rats, five rats per group*

Group	Colour	Average body weight g.	Hair growth in mm. on day 4	8	12	17
1	Albino	137	3·8	6·3	9·4	13·7
2	Fawn	159	5·6	10·2	13·4	16·2
3	Brown and white	182	6·4	11·2	15·8	18·4
4	Brown	177	5·6	9·3	12·7	16·6
			Number with full coats			
1	Albino	137	1	1	1	4
2	Fawn	159	0	0	1	5
3	Brown and white	182	0	1	2	5
4	Brown	177	0	0	0	3

The results are shown in Tables IV and V. Hair growth in the rats when young was slower than in the second test. It was also slower in the albinos of this series than in the albinos previously tested, and slower also than in the coloured rats of this series. The same seriation, albinos, fawn, brown and brown and white (hooded), in order of increasing rate of growth, is seen in each test. Testing the data for the 7th day (Table IV), when the differences between groups were maximal, we nevertheless find that only a

low degree of significance can be attached to the results, as the ratio of the variance between groups to that within groups is 3·1, giving a P of just over 0·05. If groups 1 and 4 are compared by a t test [Fisher, 1938], $t=3·3$, $P<0·02$, but as these two groups have been selected in a non-random manner, the degree of confidence with which we can assert that their means differ is again low. At no level do the mean responses in Table V differ significantly from one another. It seems, however, that the degree of variation among these mixed albino and coloured rats is such that they are unsuitable for use in tests. The incidence of full coats also varies widely from that for the Glaxo albinos, as a complete new coat may be seen in this stock as early as 3 or 4 days after shaving, but was not seen before 11 days in the Glaxo rats, or in rats from our own Wistar colony.

There was no significant correlation between body weight and rate of regeneration of hair in the mixed stock. A test of the results from which Table IV was compiled gave a correlation coefficient of 0·175 for the data of day 10, which with 16 degrees of freedom is insignificant. As these results showed more likelihood of giving a significant correlation than any others, further tests were not made. In view of the slower rate of hair growth in immature animals as compared with adults, it would be unwise to include them in a test, in which the intra-group variance is to be limited as far as possible.

Effect of gonadectomy

Albino rats of the Glaxo strain were gonadectomized when adult and their hair growth investigated as before. Twelve days after shaving, the castrated males, eight in number, average body weight 172 g., had grown 14·1 mm. of hair; and nine ovariectomized females, average body weight 154 g., had grown 12·2 mm. One of the females failed to grow any new hair before the 12th day, and if this rat is omitted from the calculations the average growth is 13·8 mm.

The t test showed no significant difference between the two groups, whether the aberrant female was omitted or not, or between the hair growth of the gonadectomized rats of either sex and that of the normal *males* previously investigated. In both groups, three rats showed full coats of hair by the 12th day, and all were fully covered by the 17th day, showing in this respect no difference from normal rats.

Gonadectomy thus increased the growth rate of hair in female albino rats to a rate indistinguishable from that of normal males, and did not affect that of males significantly.

Effect of thyroidectomy

Nine female albino rats of the Institute's Farm stock were thyroidectomized when adult, and tested 3 months later at an average body weight of 163 g. At autopsy, six of the rats were found to have no thyroid tissue remaining, and three showed traces of unremoved or regenerated tissue. By the 24th day, the six completely thyroidectomized rats had grown only 12·9 mm. of hair, as compared with a control growth of 17·5 mm. in ten controls, and only two had produced full coats. The three incompletely operated rats showed full coats by the 16th day, and had grown 19·2 mm. of hair on the 24th day, thus behaving like the controls, from which they did not differ significantly.

Thyroidectomy therefore decreases both the rate of hair growth and its completeness in female albino rats, a t test showing a significant difference between the test and control groups ($P < 0{\cdot}05$).

The results with gonadectomized and thyroidectomized rats thus gave sufficient indication that the test method is adequate for detecting experimental alteration of the rate and extent of hair regeneration. Further experiments were therefore directed to discovering the effects of various injected or inuncted substances.

As many of the substances tested were ineffective, details will be given only of the positive tests, the rest being summarized in Table VIII. Tests in which hair growth was affected by treatment comprise those with steroid hormones, and with extracts of rat pituitary glands.

Effect of oestrogens and androgens

Table VI presents a summary of simultaneous tests conducted on adult albino rats of both sexes, all from the Institute's own colony. The substances were injected, or inuncted in oil on the shaven patch, and measurements were taken during and after the 14 days of treatment. Hair growth on the 14th day is shown in the table, and the completeness of the new coat recorded for later dates.

Tested by variance ratios [Fisher & Yates, 1938] the tests with both male and female rats show significant deviations from a random distribution of the mean hair growth (ratio for males = 4·60, $P < 0{\cdot}01$; ratio for females = 3·94, $P < 0{\cdot}05$). t tests of separate groups compared with the controls show that the significant deviations are to be attributed to the males inuncted with oestrone ($t = 2{\cdot}36$, $P < 0{\cdot}05$) and the females injected with oestrone ($t = 3{\cdot}06$, $P < 0{\cdot}02$). In both these groups the administration of oestrogen causes a significant retardation of the rate of hair growth. The groups in which males were injected with oestrone, and females inuncted with oestrone, although they show less growth than their respective

Table VI. *The effect of testosterone and oestrone in oil on hair growth in the albino rat, five rats per group*

Substance	Route	Daily dose mg.	Daily volume ml.	Average body weight g.	Hair growth after 14 days mm. $\pm \sigma_m$	% full coats on 18th day	% full coats on 26th day
(*a*) *Male rats*							
Testosterone	Subcutaneous	0·25	0·5	190	19·2 ± 0·60	80	100
Testosterone	Inuncted	0·1	0·1	161	17·6 ± 0·49	80	100
Oestrone	Subcutaneous	0·1	0·5	174	17·3 ± 0·20	0	0
Oestrone	Inuncted	0·05	0·1	161	16·4 ± 0·58	0	0
Controls	—	—	—	171	18·1 ± 0·43	100	100
(*b*) *Female rats*							
Testosterone	Subcutaneous	0·25	0·5	149	16·5 ± 0·59	100	100
Testosterone	Inuncted	0·1	0·1	152	18·0 ± 0·32	80	100
Oestrone	Subcutaneous	0·1	0·5	162	15·5 ± 0·22	60	100
Oestrone	Inuncted	0·05	0·1	157	16·4 ± 0·62	60	60
Controls	—	—	—	145	16·9 ± 0·40	80	100

controls, are not significantly different from them. On the other hand, both groups of oestrone-treated males show highly significant delay in the formation of a full coat, there being bare patches on all of the rats so treated as late as the 26th day, while the controls showed full coats by the 18th day. Oestrogenized females showed only a slight delay in this respect.

The injection or inunction of testosterone did not affect hair growth in either sex. This contrasts to some extent with the findings of Zuckerman & Parkes [1939], who showed that in the castrated *Hamadryas* baboon, the injection of testosterone propionate caused a resumption of the grey coat with longer hairs which is characteristic of the normal male. In an immature drill injected with testosterone propionate, the coat also became much thicker. In these Primates, male hormone has an undoubted influence on the character of the hair, if not also on its rate of regeneration and growth. There is of course abundant evidence that hair distribution in man is dependent on the internal secretions of the testis.

In a further test, in which adult male rats had been implanted with tablets of various oestrogens and androgens, the effect of oestrogens in inhibiting hair growth was again apparent. The rats were shaved several weeks after the implantations had been made, and the hair grown after 14 days in rats carrying tablets of testosterone mono- or di-esters was 16·0 ± 1·25 mm. in ten rats, and in those carrying oestriol, oestradiol and various mono- and di-esters it was 8·96 ± 1·61 in thirteen rats. The difference is very highly significant. In the group carrying tablets of androgen, 80% had full coats on day 14, when only 15·4% of those carrying oestrogen tablets had full coats.

Oestrogens thus have a potent action in decreasing the rate and extent of hair regeneration in male rats. They decrease the rate of hair growth in females, but to a lesser extent, and this will be expected if intact females are presumed to produce endogenous oestrogens in sufficient quantities to cause almost a maximal inhibition of the growth of the hair, independent of exogenous material.

Effect of extracts of rat pituitary glands

Table VII shows the effect of two types of extract of pituitary glands from rats. A significant increase in the rate of growth of hair was obtained when a crude saline extract of female pituitaries was injected into females ($t = 2{\cdot}69$, P *ca.* $0{\cdot}02$). This extract was prepared by grinding freshly obtained

Table VII. *The effect of injected extracts of rat pituitary glands on hair growth in the albino rat. Five rats per group, controls nine rats per group*

Type of rat pituitary extract injected	Daily dose mg.	Daily volume ml.	Sex of rats	Average body weight g.	Hair growth after 10 days mm. $\pm \sigma_m$	% full coats on 18th day
Acetone-dried female glands in saline	3	1	♀	184	$12{\cdot}8 \pm 0{\cdot}95$	100
Crude saline extract of female glands	20	1	♀	162	$14{\cdot}3 \pm 0{\cdot}32$	80
Controls	—	—	♀	172	$12{\cdot}7 \pm 0{\cdot}38$	89
Crude saline extract of mixed male and female glands	20	1	♂	124	$11{\cdot}2 \pm 2{\cdot}68$	80
Controls	—	—	♂	1·16	$12{\cdot}8 \pm 1{\cdot}05$	78

glands with sand and saline, leaving them overnight in the cold store, centrifuging next morning and adjusting the *p*H to 8·5, regrinding the residue and centrifuging again, and adjusting the *p*H once more to 8·5. It was used at a concentration of 20 mg. of original tissue per ml. of saline extract.

A suspension of acetone-dried pituitary glands from female rats was ineffective in females, and a crude saline extract of mixed male and female glands—there being insufficient glands of one sex available—was ineffective in males. It will be noted in Table VII that only 3 mg. per day were given of the acetone-dried glands. This is because 3 mg. of dried glands are approximately equivalent to 20 mg. of fresh material.

The interesting result with the crude extract in females has not yet been repeated, since insufficient numbers of rats are available for preparing the extract. In any case, it was not unexpected, since the only clear-cut cases of success in stimulating hair growth in humans have been treated with

pituitary preparations. It is in fact surprising that a variety of other pituitary extracts, whether from fresh or dried material (Table VIII), have been without effect, and one may conclude that the normal rat's hair is

Table VIII. *Substances having no significant effect on hair growth in the albino rat*

Substance	Daily dose mg.	Solvent	Sex of rats	Remarks
Progesterone	1·0	Oil	♂	—
Desoxycorticosterone acetate	1·0	Oil	♂ and ♀	Subcutaneously implanted tablets also ineffective
Thyroxin	0·1	Water	♂ and ♀	—
Prolactin (ox), AP. 48. C.	2·5	Saline	♂ and ♀	Contains growth hormone
Prolactin (pig), AP. 74. C.	2·5	Saline	♂ and ♀	Contains growth hormone
Acetone-dried pig pituitary glands	100·0	Saline	♂ and ♀	—
Acetone-dried ox pituitary glands	100·0	Saline	♂ and ♀	—
Acetone-dried horse pituitary glands	100·0	Saline	♂ and ♀	—
Fresh crude extract of ox pituitary glands	250·0	Saline	♂	No effect on castrates

not capable of growing at a much greater rate than it exhibits spontaneously, particularly in males. Perhaps the response of females to the crude rat pituitary extract may be due to the fact that the growth of their hair is retarded by endogenous oestrogens, the effect of which may be overcome by additional stimulation from injections of pituitary substance.

Effect of other substances

Although thyroidectomy affects hair growth adversely, the administration of thyroxin to normal rats did not increase it (Table VIII). Progesterone, desoxycorticosterone acetate and the various pituitary extracts already mentioned were also ineffective.

SUMMARY

1. Hair growth in the rat after depilation by shaving or by barium sulphide paste was assessed by the length of the new hairs at stages during their growth, and by estimating the completeness of the new growing patch. Growth was not affected significantly in normal rats by the method of depilation, the area depilated, the side of the body used, or by previous depilation. Part of the denuded area starts to grow immediately; the rest of it does not show any new hair until between 11 and 18 days after the beginning of the experiment in adult rats, but the second growth phase occurs earlier in immature rats.

2. Male albino rats grow hair faster than females, on the average, but the hair of castrated males and ovariectomized females grows at about the

same rate, equal to that of normal males. Growth of hair in mixed albino and coloured rats, bred from the same (dealers') stock, was more variable than in albinos, which were therefore used exclusively in tests.

3. Thyroidectomized female rats exhibited less rapid and less complete hair growth than normals.

4. Administration of androgens did not affect hair growth but oestrogens slowed it, more strongly in males than in females, in which more endogenous oestrogen is presumably already at work. Oestrogens also tend to cause bare patches to persist on the shaved area.

5. Female rats exhibited increased hair growth when treated with a crude saline extract of the pituitary glands of female rats, but were insensitive to acetone-dried material from the same source. Male rats were unaffected by a crude saline extract of mixed rat pituitaries.

6. Other substances tested, and which did not affect hair growth, were progesterone, desoxycorticosterone acetate, thyroxin, and various crude and refined extracts of ox, horse and pig pituitary glands.

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THE EFFECT OF IMPLANTING TABLETS OF SYNTHETIC OESTROGENS ON THE HISTOLOGY AND CYTOLOGY OF THE ANTERIOR PITUITARY OF IMMATURE RATS

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THIS paper is a report on the histological and cytological changes observed in the anterior lobes of the hypophyses of a number of immature rats of the Wistar strain, subjected to the subcutaneous implantation of synthetic oestrogens in tablet form. A detailed account of the experimental work has already been published [Noble, 1939], and only those parts immediately relevant to this investigation will be quoted here.

METHODS

The animals were killed by having their necks broken, and the pituitaries were removed immediately, cut into two parts, and fixed in Champy's fluid. In a number of instances, indicated in Table I, pieces were post-osmicated according to the method described by Severinghaus [1932]. By this method, a number of excellent impregnations of the Golgi bodies was obtained. Several staining techniques were used, but the most satisfactory were:

(1) A combination of Altmann's mitochondria method, using a saturated solution of acid fuchsin in aniline water, followed by Mallory's aniline-blue, orange-G mixture and

(2) The acid fuchsin, methyl-green, acid-violet technique described by Severinghaus [1932].

The latter was found to be more precise for cytological detail, whereas the former gave a better gross differentiation of the chromophil cells, especially the basophils. The glands were sectioned at 3–4 μ.

Pituitaries from normal immature and mature animals were fixed, sectioned and stained in the same manner for purposes of comparison.

Table I. *Effect of implanting tablets of stilboestrol or hexadiene compound in immature rats for 10 or 17 weeks respectively and of subsequent chorionic gonadotrophin injection*

Rat	Initial weight g.	Weight increase* g.	Testis weight* mg./100 g.	Ovary weight* mg./100 g.	Pituitary weight† mg./100 g.	Degree of vascularity‡	Size of Golgi body‡	Size of mitochondria‡	No. of acidophils‡ (granulated)	No. of basophils‡ (granulated)	No. of large chromophobes (degranulated chromophils)
(*a*) *Implanted with* 10 *mg. stilboestrol tablet for* 10 *weeks*											
748	42	101	108	.	5·5	n.	+	n.	n.	− − −	+
752	46	84	146	.	10·0	+ +	+ +	+ +	−	− − −	+ +
759	41	76	.	6	18·8	+ +	+ + +	+ +	0	0	+ + +
763	38	79	.	5	12·8	+ + +	+ + +	+ + +	− − −	− − −	+ + +
(*b*) *Implanted with* 10 *mg. hexadiene compound tablet for* 17 *weeks*											
788	54	63	.	5	14·5	+	+ + +	.	− − −	− − −	+ + +
790	49	73	.	10	10·6	n.	+ + +	.	− − −	0	+ + +
792	43	64	.	8	14·0	+	+ +	.	− −	− − −	+ +
(*c*) *As* (*b*)+*total dose of* 2 *mg. U.P.* 10 *in* 5 *days before death*§											
793	41	77	.	Fibrosis	14·4	+ + +	+ + +	.	− −	0	+ +
795	46	75	.	25	17·3	+ + +	+ + +	.	−	0	+ +
(*d*) *As* (*b*)+*total dose of* 4 *mg. U.P.* 10 *in* 5 *days before death*											
796	40	68	.	31	11·1	+ + +	+ + +	.	n.	−	n.
797	40	74	.	17	7·8	+ +	+ + +	.	n.	−	n.

* The values for weight increase and gonad weight are definitely subnormal [cf. Noble, 1939].

† Normal pituitary weight = 5 mg./100 g. or less.

‡ n. = normal. +, + +, + + + = slightly, considerably and greatly *above* normal. 0 = entirely absent. −, − −, − − − = slightly, considerably and greatly *below* normal.

§ 2 mg. U.P. 10 gave ovary weight of 81 mg. in normal immature rats.

RESULTS

Table I gives a summary of the more important histological and cytological features of the anterior lobes of two male and two female immature rats implanted with 10 mg. tablets of stilboestrol or 4:4′-dihydroxy-α:β-diethylstilbene [substance 27 in Noble, 1939] over a period of 10 weeks. With the exception of rat 748, the pituitaries showed a marked enlargement which was especially noticeable in rat 759. The pituitary of rat 748 presented an almost normal histological picture, except for the extensive degranulation of the basophil cells, and the increase in chromophobes; some of the large chromophobes, from the position and form of their Golgi bodies, being degranulated basophils. The remainder of the pituitaries all showed, in a greater or lesser degree above the normal, an increased vascularity, a generalized enlargement of the Golgi bodies, and mitochondria (Plate I, figs. 1, 2), and an increase in particular of the large chromophobes. The latter are clearly to be interpreted as degranulated chromophils, as Severinghaus [1936, 1937], Kirkman [1937] and others have shown. As will be seen from Table I all four pituitaries showed that the oestrogenic substances had brought about a degranulation of the basophils, and in two instances (759 and 763) this was complete. The changes in the acidophil cells were of the same nature, but the extent of the effect was rather more varied, with 748 presenting an almost normal acidophil picture and 763 showing complete degranulation.

Another series of immature female rats had been implanted over a period of 17 weeks with 10 mg. tablets of 4:4′-dihydroxy-γ:δ-diphenyl-β:δ-hexadiene [substance 26 in Noble, 1939]. During the 5 days before death, two animals of the series had been injected with a total of 2 mg. chorionic gonadotrophin (pregnancy urine extract U.P.10), and another two with a total of 4 mg. of the same substance; these results are also summarized in Table I.

The histological and cytological appearances of the pituitaries of the rats which had not been injected with chorionic gonadotrophin were comparable with those treated with stilboestrol (Plate I, fig. 3). Again, the important characteristics were:

(1) Hypertrophy of the Golgi bodies.

(2) Varying degrees of degranulation of the chromophil cells, that of the basophils being almost complete.

(3) A marked increase in the proportion of chromophobes, particularly of large chromophobes.

The pituitaries of the rats injected with pregnancy urine, while showing the increased weight and hypertrophied Golgi apparatus of those treated

with oestrogens alone, gave evidence of important changes in the cellular composition of the gland. These changes consisted in the reappearance of the chromophil elements correlated with a relative decrease in the chromophobe constituents. As will be seen from Table I, this recovery was most complete in the animals treated with the higher dosage of pregnancy urine. An examination of Plate I, fig. 4, shows that the histological appearance presented is comparable with that of a normal gland in regard to the relative abundance of the cellular constituents. The important question as to whether the physiological activity of such glands is comparable with that of normals is discussed below.

DISCUSSION

The interpretation of cytological changes seen in an organ as the result of any particular experimental treatment can only be made with any degree of certainty when the significance of the phase of the normal life cycles of its constituent cells has been established. This is particularly true of the secretory cells of glandular organs, where, in addition to generalized changes in cellular morphology, there must also be considered the more specific alterations which occur among the cell parts. Included under the latter are the temporary cell parts such as the so-called 'pro-secretion' granules and the permanent cell parts like the nucleus, Golgi body and mitochondria.

Before any attempt to correlate cytological characteristics with physiological activity in the anterior lobe can be considered, two assumptions must be made:

(1) That the specific granules of the chromophil cells are the precursors of the active principles of the gland, and

(2) That these cells in all their phases are in a state of dynamic equilibrium with each other.

As yet, there is nothing of a histochemical nature to support the first assumption, and its validity can only be based on indirect evidence obtained from the behaviour of similar granules in other glands, such as the salivary glands, pancreas, etc., after stimulation [Bowen, 1929]. So far, however, it has proved satisfactory as a working hypothesis.

The second assumption has more justification, since a definite secretory cycle in the anterior pituitary cells of certain mammals has been convincingly established by Severinghaus [1936] for the rat, and Kirkman [1937] for the guinea-pig. This secretory cycle consists fundamentally of a regular accumulation and release of specifically stainable granules by the chromophil cells, the effete cells being replaced by the growth and differentiation of the small chromophobes. In consequence, any 'time slice' of

the gland will show cells at varying stages of their secretory cycles. There will be cells, as yet inactive (small chromophobes), cells fully charged with granules (granulated chromophils), cells in the process of degranulation (degranulating chromophils), and cells which have discharged their granules (large chromophobes). In order that there may be a more or less uniform release of secretions from the gland as a whole, it is obvious that at any given time the relative proportions of cells at any particular stage of the secretory cycle must be approximately constant, provided the individual *rates* of granule accumulation and release be the same. That something in the nature of such a dynamic equilibrium exists, is shown by the fact that in normal animals the relative proportion of the cells in their different cyclic states is fairly constant. That the position of equilibrium, as indicated by a change in histological composition, may temporarily shift, as in ageing, oestrus, pregnancy, etc., has been well established [Wolfe & Cleveland, 1933; Wolfe, 1935; Chadwick, 1937; Kirkman, 1937]. These observations, however, do not run counter to the general idea of an equilibrium.

From what has been said above, it is clear that the concept of a 'normal' gland from the histological point of view is of great importance. Although it is evident that any unspecified change in the physiological activity of the gland might be inferred from accompanying changes in its histological configuration, it is equally evident that a consistent histological picture over a period of time may conceal considerable fluctuations in activity, since a uniform increase or decrease in the rate of formation and release of secretion will not necessarily be reflected in an alteration in the histological composition of the gland.

In view of the often uncertain correlation between the histological appearance and physiological activity of gland cells in general, it has been necessary to find out whether any of the permanent cell parts will function as indicators of the activity of the labile cell parts such as 'pro-secretion granules'. In other words, the problem has become a cytological one.

Of the various structures which have been used as indicators of changes in secretory activity in cells, the Golgi bodies have proved most satisfactory. There has been produced a considerable body of evidence which indicates that any increased activity of glandular cells is associated with some degree of hypertrophy of their Golgi bodies [Ludford & Cramer, 1928; Bowen, 1929; Okkels, 1932; Kirkman & Severinghaus, 1938; Welch & Broders, 1940]. What is lacking, however, is evidence which indicates whether the hypertrophy is to be correlated with an increase in the rate of production of the secretion, an increase in the rate of its release, or both.

It will have been seen from Table I that hypertrophy of the Golgi bodies of the anterior lobe cells, as observed by direct impregnation with osmic

acid or by their negative image, was a characteristic feature of all the experimental rats with the exception of 748. This has been interpreted as evidence of a greatly increased activity on the part of the glandular cells, a fact supported in addition by instances of vacuolation in the Golgi area and cytoplasm and also an increase in number and an enlargement of the mitochondria.

In contrast to the cytological picture of heightened activity is the undoubted fact that the gonadotrophic potency of the gland has been depressed, as evidenced by the infantile state of the gonads and, as was first shown by Meyer, Leonard, Hisaw & Martin [1930], a lowered gonad-stimulating potency on implantation. This apparent contradiction may be resolved by the assumption that the action of the oestrogens in excess is to stimulate the release of gonadotrophins and depress the rate of their secretion. The net result is that output rapidly exceeds synthesis and the cells enter a state of physiological exhaustion, the cytological signs among others being a permanent degranulation of the chromophils and hypertrophy of the Golgi apparatus.

With the exclusion of the animals treated in addition with chorionic gonadotrophin, Table I shows that the effect of the oestrogens was on the whole most marked in the basophils, which appeared to be more susceptible to their stimulatory influence than the acidophils, although the latter were in a number of instances so completely degranulated that the pituitaries had a completely chromophobic aspect. In some cases the fact that the Golgi hypertrophy was generalized, extending to the small chromophobes, suggested that the oestrogens had initiated a precocious secretory cycle in the cells. In some of these cells scattered acidophil granules were still present. Such a differential degranulation after oestrone treatment, depending to a large extent on dosage, has been recorded by a number of investigators including Nelson [1934] and Wolfe & Wright [1938].

As was shown by Noble [1939] the response to oestrogens of immature rats differs from adults in that there is only a slight retardation of body growth up to 100–120 g. This might be correlated with the greater resistance of the acidophils to the action of oestrogens, as evidenced by their greater relative frequency compared with the basophils, growth only ceasing when there is a generalized 'secretory exhaustion' of the cells.

It has been remarked previously that the effect of chorionic gonadotrophin was to initiate a regranulation of the chromophilic elements, with the result that in two instances (796 and 797, Table I) the anterior lobes presented a practically normal appearance. This suggests that the gonadotrophin of the urine stimulated the secretory phase of the cells with a consequent regranulation, first of the acidophils and then of the basophils. A similar antagonistic effect between oestrogens and anterior pituitary-like

substances on the histology of the anterior pituitary has been reported by Wolfe [1937], who showed that the injection of such substances simultaneously with oestrone tended to nullify the oestrone effect.

Noble [1939] showed that whereas typical corpora lutea were produced by pregnancy urine extract after a short period of crystal implantation, in this instance only a diffuse luteinization with one example of typical corpora lutea formation occurred after the prolonged implantation. In this respect the effect was similar to that produced by chorionic gonadotrophin on hypophysectomized animals. It appears therefore that in these experiments the oestrogens deranged the pituitary function, with the result that the animals reacted as if they were hypophysectomized. The relatively normal histological appearance of the pituitaries coupled with the cytological evidence of increased cellular activity suggests that a more prolonged administration of chorionic gonadotrophin might have resulted in a complete restoration of the secretory cycle of the cells, the gonadotrophins then released producing follicular development and luteinization of the ovary.

SUMMARY

1. With one exception, a state of hypersecretion amounting to secretory exhaustion was produced in the anterior pituitary of immature rats implanted subcutaneously with tablets of 4:4′-dihydroxy-γ:δ-diphenyl-β:δ-hexadiene and stilboestrol. This was evidenced by a degranulation of the chromophils, an increase in the chromophobes (particularly the large chromophobes), hypertrophy of the Golgi bodies and vacuolation of the cytoplasm of the cells.

2. The acidophils showed a greater resistance to the stimulatory effects of the oestrogens than the basophils, the latter showing the greatest extent of degranulation. The former observation is perhaps to be correlated with the slight retardation in body growth up to 100–120 g. of these animals as compared with adults.

3. The pituitaries of rats injected in addition with chorionic gonadotrophin, during the 5 days before death showed that although the heightened cellular activity was maintained, there was definite evidence of a regranulation of the chromophil cells, with a consequent approach to a normal histological picture.

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DESCRIPTION OF PLATE I

FIG. 1. Anterior pituitary of immature female rat (no. 759) after implantation for 10 weeks with a 10 mg. tablet of stilboestrol. (1) Two large chromophobes, showing vacuolated negative image of Golgi apparatus to left of nucleus. The upper cell shows a cluster of enlarged mitochondria to left of nucleus. (2) Large chromophobe (probably a degranulated basophil) showing detached negative image of Golgi with mitochondria to right of the nucleus. (3) Large chromophobe (probably a degranulated acidophil) showing enlarged Golgi body abutting on right side of the nucleus. (4) Greatly thickened connective tissue. Photomicrograph. Champy-Mallory. ×400.

FIG. 2. As fig. 1, impregnated with osmic acid to show Golgi bodies. (1) Hypertrophied Golgi bodies. (2) Hypertrophied Golgi bodies of degranulated basophil cells. Note that the Golgi is detached from the nucleus. (3) Hypertrophied Golgi body of a degranulated acidophil cell. Note that the Golgi caps the nucleus. Photomicrograph. Champy-osmic acid-Severinghaus. ×400.

FIG. 3. Drawing of part of anterior lobe of an immature female rat (no. 788), implanted for 17 weeks with a 10 mg. tablet of hexadiene compound. (1) Degranulating acidophil cell. (2) Degranulated basophil cell with hypertrophied Golgi body (large chromophobe). (3) Degranulated acidophil cell with hypertrophied Golgi bodies (large chromophobe). (4) A large chromophobe with enlarged mitochrondria cupping another chromophobe with an hypertrophied Golgi apparatus. (5) Greatly thickened connective tissue. (6) Small chromophobe. Champy-osmic acid-Mallory. ×600.

FIG. 4. Anterior pituitary of an immature female rat (no. 796) implanted for 17 weeks with a 10 mg. tablet of hexadiene compound and injected for the 5 days preceding death with a total of 4 mg. U.P. 10 (chorionic gonadotrophin preparation). (1) Fully granulated acidophil cells. (2) An abnormally small acidophil cell, probably a precociously granulated small chromophobe. (3) Fully granulated basophil cell. (4) Large chromophobes, the lower shows an hypertrophied negative image of its Golgi body to the right of the nucleus. (5) Small chromophobe. (6) Dilated sinusoids containing red blood cells. Photomicrograph. Champy-Mallory. ×400.



Fig. 2



Fig. 4



Fig. 1

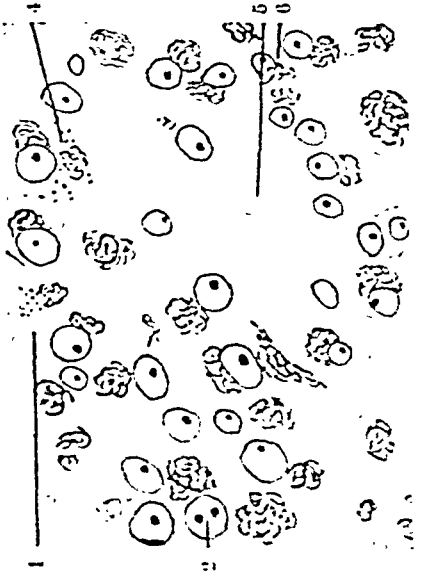


Fig. 3

THE BEHAVIOUR OF THE UTERUS OF THE RHESUS MONKEY UNDER THE INFLUENCE OF CERTAIN HORMONES

By G. H. BELL

From the Institute of Physiology, the University of Glasgow

(*Received 18 October 1941*)

In view of the well-known species differences in the realm of reproductive physiology it would be a great advantage if an animal were found whose uterine muscle reacted in a manner similar to that of the human subject. It would make investigations of dosage of hormones and of hormone therapy of uterine muscle distinctly easier. Apart from some work on the activity of the Fallopian tubes of monkeys by Seckinger & Corner [1923] *in vitro*, and by Westman [1929] *in vivo*, no investigation of the movements of the monkey uterus *in vivo* has been noted by Reynolds [1939] in a very comprehensive review. The present work was carried out on the most readily available monkey, the rhesus monkey. Unfortunately, well-grown specimens are difficult to get from the dealers; the animal is also rather expensive for any routine investigation. Until its possibilities have been explored, however, its usefulness cannot be defined. A review of some of the similarities and differences in reproductive phenomena between man and monkey has already been given by Hartman [1939].

METHODS

The fifteen animals, all of the species *Macaca mulatta*, were healthy but probably subadult, except M 11, which had the wrinkled sex skin of the adult, and M 3 which showed an ovulating follicle at operation although the sex skin was not wrinkled; M 3 and M 11 were the heaviest animals in the series. It is not possible to give an estimate of the age of any of the animals, but, as will be seen later, there is in the experiments no characteristic of the behaviour of the uterus which can be correlated with the body weight (as an index of the age) of the animals.

All the animals were ovariectomized through a mid-line ventral incision using aseptic precautions. Nembutal (30 mg./kg.) given subcutaneously in about 5 ml. of Locke's solution was found to be an ideal anaesthetic. Only a little ether by inhalation was necessary when going through the peritoneum. The abdomen was closed carefully, layer by layer, and the skin wound covered by collodion gauze. In order to prolong the period of post-operative quiescence a further dose of nembutal was given when the animal

showed signs of recovery. No further treatment was required, and the wounds healed well in spite of the great activity of the animals. No injection of hormones was given until 10 days after this operation.

The subsequent treatment of the animals is given in detail in Table I. The first group received no treatment at all, the second oestrone in sesame oil only, the third oestrone and testosterone, and the fourth oestrone and progesterone. It will be seen that the time from spaying to the recording of the uterine movements is either 21, 26 or 32 days. In this way the diminution of the effect of the oestrone with time is allowed for, and any one group may be regarded to a certain extent as a control group to the others.

At the time of the final observations on the movements of the uterus the animals were kept under light nembutal anaesthesia supplemented by ether during the preliminary procedures of cannulation. The abdomen was opened near the original wound and the uterus exposed; a boat-shaped cannula [Bell & Robson, 1936] was attached to it by stitches in the fundus and cervix, the central stitch being taken over two pulleys to a lever writing on a smoked drum. The cannula was 2·8 cm. in length (actually the size used previously for the mouse), and because of the small size of the uterus and its fibrous character it was necessary to use a lever magnification of 35 to obtain an average excursion of 2 cm. The abdomen was closed around the funnel of the cannula; a leg vein was cannulated. The temperature was maintained throughout at 38° C.

When a satisfactory sample of the spontaneous activity of the uterus had been recorded a graded series of injections of oxytocin (specially purified 'Pitocin') was given intravenously in about 0·5 ml. of Locke's solution followed by about 0·5 ml. of Locke's solution to clear out the cannula and the vein. When the threshold dose of oxytocin had been found a small dose of vasopressin (usual commercial standard of 'Pitressin', Parke, Davis and Co.) was injected. When the effect of this had passed off the uterus was quickly removed by cutting through the broad ligament and the vagina; Michel clips carrying cotton threads were clipped to the fundus and the cervix, two strips were quickly cut off and immediately suspended in oxygenated Locke's solution in 60 ml. containers in a thermostatically controlled bath (38° C.). When the action of oxytocin had been recorded the strips were fixed for histological examination.

RESULTS

The results of the experiments are given in detail in Table I, and typical tracings from each group are given in Figs. 1–4. It will be seen at once that the behaviour of the uteri within any one group is variable, and the findings in any one group overlap those in any other to such an extent that

Monkey no.	Wt. kg.	Treatment beginning 10 days after ovariectomy	Time from ovariectomy* to end of exp. days	Threshold dose of oxytocin units	Vasopressin dose and effect* units	Wave duration min.	Wave height cm.	Threshold dose of oxytocin *in vitro*
M 3	4·15	None	21	0·05	—	2·0	2·4	0·5 and 1·0
M 6	2·3	None	21	0·1	0·1 R	0·8	0·5	0·2 and > 1·0
M 1	2·0	10 mg. oestrone over 7 days; no treatment next 4 days	21	0·01	—	0·6	1·8	?1·0
M 4	3·5	As M 1	21	0·3	—	0·8	2·0	?1·0
M 11	4·0	10 mg. oestrone over 10 days; no treatment next 6 days	26	0·05	0·2 N	1·4	1·0	?1·0
M 14	2·0	5 mg. oestrone over 10 days; 300 mg. testosterone propionate over next 12 days	32	0·01	0·2 small R	0·4 (6·0)	0·4 (2·0)	—
M 15	2·9	As M 14	32	1·0	0·1 R	0·8 (3·0)	0·6 (2·0)	—
M 16	2·6	As M 14	32	0·02	0·1 R	0·3	1·0	—
M 2	2·8	10 mg. oestrone over 7 days; 10 mg. progesterone over 5 days beginning last day of oestrone treatment	21	0·025	—	5·0	2·0	1·0
M 5	3·5	As M 2	21	0·5	0·1 R	6·0	2·5	1·0
M 7	2·5	10 mg. oestrone over 10 days; 21 mg. progesterone over 7 days beginning last day of oestrone treatment	26	0·1	0·1 N	2·0	1·5	0·1
M 8	1·9	As M 7 but 35 mg. of progesterone	26	> 0·05	—	2·5	3·0	—
M 10	2·0	As M 7 but 70 mg. of progesterone	26	0·01	0·1 R	0·25 (6·0)	0·2 (1·0)	?1·0
M 12	3·8	5 mg. oestrone over 10 days; 60 mg. progesterone over 12 days beginning last day of oestrone treatment	32	0·05	0·1 R	6·0	4·0	0·1
M 13	2·0	4 mg. oestrone over 10 days; 90 mg. progesterone over 12 days beginning last day of oestrone treatment	32	0·01	0·1 R	7·0	3·0	0·05

The data for the wave duration and height are average values from the first part of the tracing. The figures in brackets indicate that waves of these larger dimensions are occasional but quite prominent in the early part of the tracing.

* N = no effect: R = relaxation.

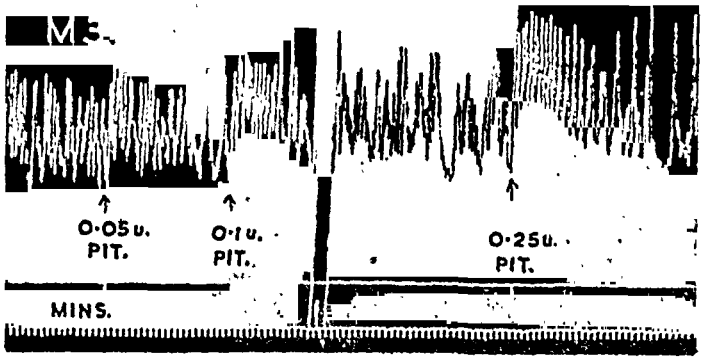


FIG. 1. M 3, no treatment. Uterine movements *in vivo*. Reaction to 0·1 and 0·25 unit oxytocin, but not to 0·05 unit. The break in the middle of the tracing was accidental.



FIG. 2. M 4, injected with oestrone. Uterine movements *in vivo*. Reaction to 0·3 unit oxytocin. Reaction to 0·1 unit doubtful because there is no rise in tone. At *BL* the base-line was raised.

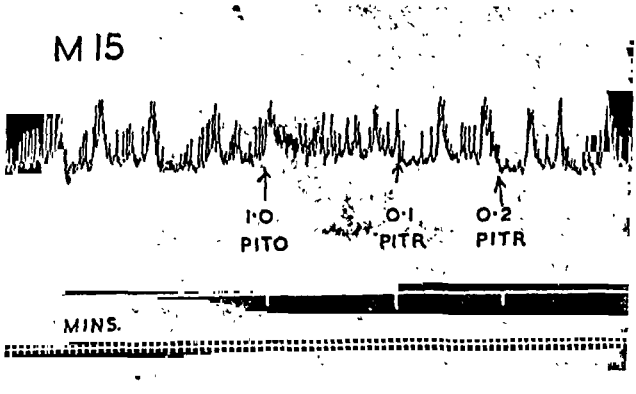


FIG. 3. M 15, injected with oestrone and testosterone. Uterine movements *in vivo*. Reaction to 1·0 unit oxytocin. Relaxation produced by vasopressin.

no separation is possible. The uteri of all groups showed spontaneous activity *in vivo*; those treated with testosterone showed perhaps less activity than the others, but even they showed occasional large waves of the extent and duration found in the other groups; the progesterone group on the whole gave slower waves than the other three groups. There was a considerable scatter of the threshold dose of oxytocin with a range of 0·01–1·0 unit; none of the treatments appeared to alter the threshold. In eight out of the ten cases in which it was tried 0·1 unit of vasopressin produced a relaxation. None of the uteri showed any spontaneous activity *in vitro* before testing with oxytocin, but after the bath had been washed out once or twice very small movements appeared; the threshold dose was 1·0 unit or more in eight out of eleven cases. At first these findings led to

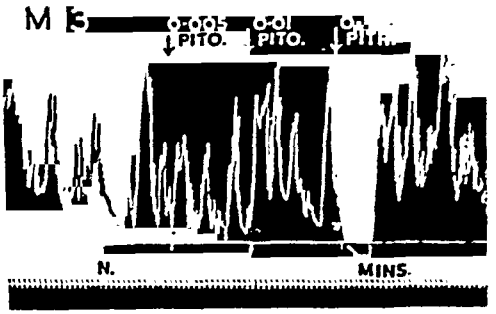


FIG. 4. M 13, injected with oestrone and progesterone. Uterine movements *in vitro*. Threshold reaction to 0·01 unit oxytocin. Relaxation after 0·1 unit vasopressin. At *N* nembutal given subcutaneously.

the suspicion that the uterine muscle was completely unreactive *in vitro*; this is not so, since histamine in a concentration of 1 in a million in the bath (in the cases of M 1 and M 2) produced a contraction. Vasopressin (0·1 unit) tried out *in vitro* in two cases was without effect.

The histological findings are, of course, well known and were only of interest as a check on the efficacy or otherwise of the various treatments. In the spayed untreated group the endometrium was atrophied and the glands were short and straight; in the oestrone-treated group the endometrium was thick and the glands long and curving; in the oestrone-testosterone group the endometrium was nearly as thin as in the first group and the glands were straight; the progesterone group showed the usual characteristic irregularity of the outline of the glands of the premenstrual stage—M 13 would be described as late premenstrual and the others as early premenstrual.

Another useful but not more than qualitative test of the efficacy of the various hormone treatments was the reaction of the sexual skin The spayed untreated animals showed no alteration of the pubic area, but all the oestrone-treated animals showed at the end of the experiment a very marked oedema of this area. When only a small dose (10 mg.) of progesterone was given the sexual skin showed either very little or no swelling; with larger doses of progesterone the initial swelling produced by oestrone disappeared before the experiment was concluded. In the testosterone group the initial oedema produced by the oestrone had not quite completely disappeared when the uterine movements were recorded.

In considering the results it should perhaps be remembered that these were obtained on somewhat immature animals; the results might have been different if older animals had been used, but against this is the fact that the results obtained on the two mature animals fit in well with the others.

DISCUSSION

The question of the dosage of the hormones to be given in an experiment where only a small number of experiments can be done for reasons of expense and supply had to be carefully considered. While small doses of hormones have been shown to produce endometrial changes [Hisaw & Greep, 1938] higher doses are used to produce bleeding on withdrawal of oestrone [Zuckerman, 1937*a*]; also relatively large doses are given in human therapeutics. These considerations led to the adoption of an average dose of about 1 mg. of oestrone in oil per day; this amount should give a good contrast with the spayed untreated group. The dosage of testosterone propionate was of the same order as that given by Geist, Salmon & Gaines [1938] to women—again a large dose should produce a change if one was forthcoming. The dosage of progesterone varied over a range, on a weight basis, from about the same as that given to women threatened with abortion to a very much greater dosage.

Although there is no doubt that the hormone dosage was sufficient as judged by the endometrial and the sex-skin changes and by comparison with the therapeutic dosage, yet the activity and the reactivity of the uterine muscle were unaffected. Because there has been so much controversy as to the effects of the corpus luteum hormone on the human uterus, seven experiments of the present series were devoted to testing out the effect of a large range of dosage of progesterone after an initial course of oestrone. In M 12 and M 13 the quantity of oestrone administered was reduced to get a higher progesterone/oestrone ratio. There is no evidence that progesterone alters the activity of the uterus or its reactivity to oxytocin. Knaus [1934] claimed that during the luteal phase of the human menstrual cycle, the uterus did not respond to oxytocin, but later workers

[including Moir, 1934; Kurzrok, Wiesbader, Mulinos & Watson, 1937 and McLellan, 1940] found no alteration in the response to oxytocin throughout the cycle. The experiments of these workers and others have been criticized by Reynolds [1939] on the ground that they used a pressure within the uterus which was much above the value of 20 mm. Hg used by Knaus, and that the foreign-body effect of the intra-uterine bag would be greatly increased. The present experiments in which the uterus was not distended fall into line with the majority of observations on the human uterus.

It can hardly be said that oestrone-testosterone treatment is an imitation of any known physiological process, but since testosterone has been used in the treatment of several conditions including dysmenorrhoea it would be interesting to know its effect on uterine muscle. The theory that dysmenorrhoea is produced by a muscular spasm receives some support from Moir [1936], who showed that pain and uterine contraction were related though not simultaneous, but Wilson & Kurzrok [1938] claim that patients with dysmenorrhoea show the same type of contraction waves as those with painless cycles. In the three monkeys treated with testosterone the uterine waves were perhaps smaller and more rapid than in the other groups, but occasionally larger and slower waves were seen; it is just possible that a small reduction in amplitude might be valuable from the therapeutic point of view. It is worth while to point out here how far observations on the rabbit differ from those on the monkey; Robson [1937*b*] reported that testosterone made the uterus *in vivo* unreactive to oxytocin and produced slight progestational changes in the endometrium [see also Klein & Parkes, 1937].

The histological changes found in the present series after administration of testosterone are the same as those described for the human subject by Geist *et al.* [1938], who suggest that the regressive changes are the 'end results of a primary inhibition of the gonadotrophic factors of the hypophysis' with consequent failure of secretion of the ovarian hormones; in the light of the present work on the monkey the endometrial changes may be due either to a direct action of testosterone or to its neutralization of the oestrone—in the intact animal these effects may be reinforced by pituitary inhibition. Loeser [1938] reported endometrial atrophy in a woman after testosterone therapy; Foss [1938] said that in his cases after testosterone the endometrium was of the interval type. In monkeys, however, previous observers [Zuckerman, 1937*b*; Engle & Smith, 1939] have not reported endometrial atrophy where oestrogen treatment was followed by a course of testosterone treatment. More recently, Hartman [1940] in a comprehensive series of experiments on monkeys did not find any evidence of antagonism between oestrogen and testosterone, although the dosage and duration of testosterone treatment were not so very different from those employed in the present work.

The action of vasopressin on the non-gravid human uterus *in vivo* was first noted by Moir [1936] and was later worked out in a quantitative manner by McLellan [1940], who showed that the oxytocic effect of 'Pituitrin' and even of oxytocin (specially purified 'Pitocin') was due entirely to their vasopressin content. In the present experiments vasopressin almost invariably produced a relaxation; it produces the same effect *in vivo* on the rabbit uterus [Robson, 1937*a*] and on the cat uterus [Robson & Schild, 1938], but no effect on the guinea-pig or hamster uterus *in vivo* [Bell, unpublished]. The human uterus seems to be an exception in its reaction to vasopressin.

The results obtained when the monkey uterus was studied *in vitro* furnish yet another example of the discrepancy between the behaviour of the uterus *in vivo* and *in vitro* already noted by Bell & Robson [1936] in the guinea-pig, Robson & Schild [1938] in the cat, and Bell [1941] in the pregnant guinea-pig. Because of this discrepancy we cannot apply the findings of Robson [1933] on strips of the human pregnant uterus *in vitro* directly to *in vivo* conditions—this is rather unfortunate because there are only a few scattered and incomplete observations on the human pregnant uterus *in vivo* and there is little likelihood of this gap in our knowledge being filled soon.

In spite of anaesthesia the uteri of the monkeys all showed spontaneous activity *in vivo*; it is rather difficult to see why they should show no movements when changed to a bath with the minimum of delay. Pregnant human uterine strips removed from women under general anaesthesia show good contractions even when there is a considerable delay in putting them into the bath of Locke's solution [Robson, 1933]. Kurzrok [1938] found that some strips taken from the non-pregnant human uterus showed no activity at all and no explanation for this could be offered; he discarded all such strips as they were not 'alive'. It seems safer to regard quiescence as one extreme of spontaneous activity and to test for viability by noting the reaction to stimuli (e.g. histamine)—the ability to react to a stimulus seems a more fundamental attribute of life than movement. The fact that human uterine strips *in vitro* usually show movements and, especially late in pregnancy, react to small doses of oxytocin, whereas the non-pregnant monkey uterine strips *in vitro* do not do either, is another discrepancy for which no explanation can be offered. There is no information available about the behaviour of the pregnant monkey uterus *in vitro*.

SUMMARY

The movements of the uterus of the rhesus monkey have been recorded *in vivo* and *in vitro* under the influence of various hormones.

After removal of the ovaries the animals received either (1) no treatment

at all, or (2) injections of oestrone in oil, or (3) oestrone followed by testosterone, or (4) oestrone followed by progesterone.

The spontaneous activity and the reactivity to oxytocin *in vivo* were both variable within any one group. Although the contraction waves tended to be small in group 3 and slow in group 4, there is practically no difference in the behaviour of the uteri of the four groups. Vasopressin almost always produced a relaxation.

None of the uteri showed any activity *in vitro* at first, and nearly all were comparatively unreactive to oxytocin although (in two cases) they reacted to small quantities of histamine added to the bath.

The similarities and the differences between these findings and the recorded behaviour of the human uterus are discussed.

I have to thank Professor E. P. Cathcart for his interest in this work, and Professor Noah Morris for allowing me to house the animals in his department. The cost of the animals was defrayed by the Rankin Research Fund of the University of Glasgow, the remainder of the expenses by the Medical Research Council. I am indebted to Messrs Schering for a very generous supply of Proluton (progesterone) and to Messrs Ciba for a large amount of Perandren (testosterone propionate). Dr White of Parke, Davis and Co. kindly supplied a quantity of specially purified Pitocin.

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THE ASSAY OF HYPOPHYSEAL GROWTH-PROMOTING EXTRACTS EMPLOYING RATS TREATED WITH DIETHYLSTILBOESTROL

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The discovery of the powerful synthetic oestrogen diethylstilboestrol [Dodds, Golberg, Lawson & Robinson, 1938] was quickly followed by the demonstration [Noble, 1938*a*, 1939*a*] that treatment with this material produces, in rats, the signs of apparent pituitary dysfunction (e.g. atrophy of the gonads and accessory sex organs, and inhibition of growth) associated with excessive treatment with natural oestrogen in this species. Noble [1938*b*, 1939*b*], applying the technique introduced by Deanesly & Parkes [1937], showed that the implantation of crystals or tablets of pure diethylstilboestrol could also induce alterations in the rate of body growth, and in endocrine function. The action of diethylstilboestrol with respect to the gonads was to some extent reversible, in that after treatment with the synthetic oestrogen the ovary of the female rat could still respond to the influence of gonadotrophic preparations.

Dr A. S. Parkes, F.R.S., suggested to us that a relatively simple test object for the assay of pituitary growth-promoting extracts might be provided by the rat whose growth had been inhibited by the implantation of tablets of solid diethylstilboestrol. Such a preparation would have obvious advantages, in that the technique of implanting tablets is much easier than that of hypophysectomy, and young rats of either sex could be used. The latter point would be of importance in a laboratory in which hypophysectomized rats were not easily available, as the usual method of assay employing intact rats requires adult female animals whose weight curves have risen to a 'plateau' at 170–200 g., of which the supply is necessarily limited.

We have accordingly investigated the possibility of using, for the assay of growth hormone, the young male rat stunted by the implantation of tablets of the artificial oestrogen. Increase in body weight, and elongation of the tail length as an indicator of skeletal growth [Freud & Levie, 1938; Freud, Levie & Kroon, 1939], have been compared as criteria of growth,

[1] Work carried out during the tenure of a Science Research Scholarship of the Royal Commission for the Exhibition of 1851.

both with the proposed new technique, and with the classical methods, which employ the adult normal female, or the hypophysectomized rat [Evans & Simpson, 1931].

METHODS

Animals. The animals used in this investigation were Wistar-strain albino rats bred in this laboratory. They received a diet of Purina Fox-Chow and water *ad lib.*

Hypophysectomy was carried out by one of us (M. G.) by the retropharyngeal method, using avertin anaesthesia. The criteria of completeness of removal of the gland are those previously described [Griffiths, 1941].

Implantation of tablets. Two 15 mg. tablets of diethylstilboestrol were implanted subcutaneously into the backs of adult male rats. The tablets were sometimes removed at the end of the experiment and weighed, in order to determine the amount of substance absorbed. In some experiments the control animals were treated similarly with tablets of an inert substance (cholesterol), or were subjected to a dummy operation. As these procedures appeared not to influence the subsequent behaviour of the animals, their employment has not been specifically noted.

Measurement of tail length was carried out on skiagrams, as described by Freud *et al.* [1939].

Anterior pituitary extracts were prepared as previously described [Young, 1938], from fresh hypophyses brought to the laboratory in a frozen condition, by extraction with saline in the cold at *p*H 8·5. The extract was so standardized that 1 ml. contained the material extracted from 250 mg. of fresh tissue (approximately equivalent to 50 mg. of dried tissue). The extract was found to contain about 20 mg. of organic matter in each millilitre. In a few experiments extracts were prepared from hypophyses other than those of the ox, but unless otherwise indicated, the preparation used in any experiment had been extracted from ox pituitary glands.

General procedure of experimentation

All extracts were injected subcutaneously.

Normal animals. After a suitable preliminary period, groups of 5–10 female rats were treated daily with extract for 2 weeks. A group of control animals was included, this being either untreated, or injected daily with an extract of liver or thymus, which had been prepared in a manner similar to that for pituitary tissue. The animals were weighed daily throughout the experiment and the average increase in weight, per rat for a group of rats during the period of 14 days, is given as the 'gross increase' in weight; the 'net increase' is the difference between the average total increases of the

experimental and control groups. In one experiment with normal animals the increase in tail length was determined simultaneously with the increase in weight.

Operated animals. For approximately 2 weeks after the operation (hypophysectomy or implantation of the tablet) the animal was untreated, but weighed daily. In some instances, the tail length was measured skiagrammetrically on the day after the operation. At the end of the preliminary period the animals were treated daily with extract for 14 days, and the increase in weight or tail length during this period determined. In a number of experiments the control groups were treated with inactive tissue extracts.

RESULTS

Increase in body weight as a criterion of growth

Normal female rats

The results summarized in Table I indicate that little difference was observed in net response whether young adult or heavier female animals of about 180 g. were used [cf. Chou, Chang, Chen & van Dyke, 1938].

Hypophysectomized male rats

Although the gross increase in weight observed was of the same order as that with normal female rats (Table I), the net gains for the treated rats were definitely greater, because the control hypophysectomized rats lost

Table I. *Influence of ox anterior pituitary extract on the body-weight increase of adult normal female rats and of hypophysectomized male rats*

Animals	Dose of pituitary extract injected daily (mg. equivalent of dried tissue)	Total no. of animals treated	Average body weight in g.		
			Initial	Gross increase in 14 days	Net increase in 14 days
Normal female rats	6·25	10	137	25	20
	12·50	15	154	41	33
	25·00	60	181	38	32
	25·00	40	121	54	31
Hypophysectomized male rats	0·00	16	133	− 9	—
	6·25	17	131	24	33
	50·00	39	111	51	60

weight during the experimental period. The relative sensitivities of the hypophysectomized and the normal rat for the assay of growth hormone have been discussed by Chou *et al.* [1938], who conclude that the former is about twice as sensitive as the latter, in terms of the percentage increase in weight.

Animals carrying tablets of diethylstilboestrol

In experiments on a total of fifty-five rats the average rate of absorption from two subcutaneously implanted tablets of 15 mg. of diethylstilboestrol was found to be 0·26 mg./rat/day (max. 0·42; min. 0·14).

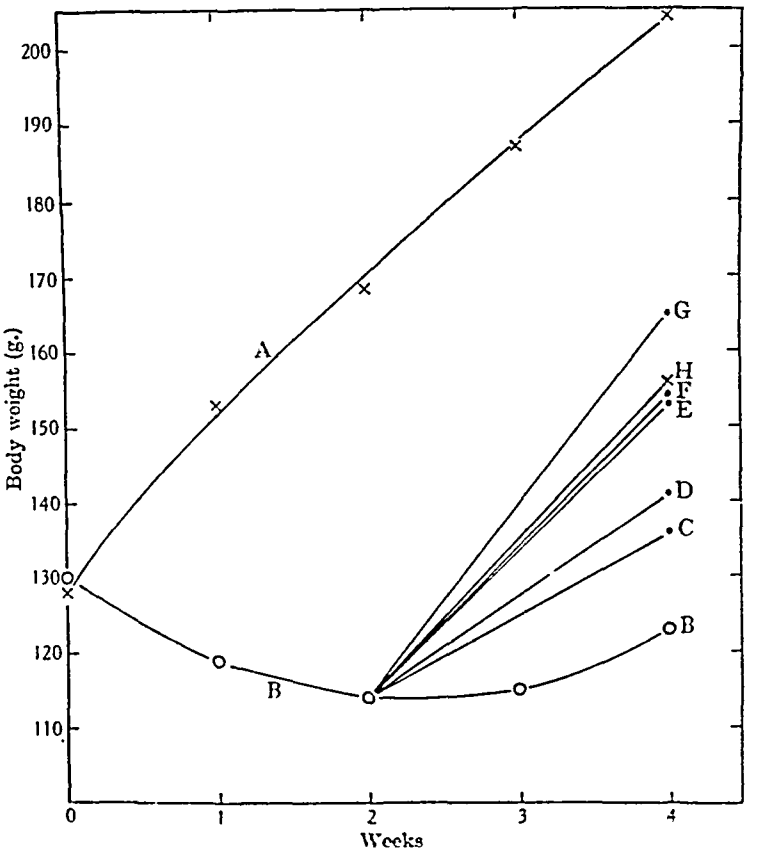


FIG. 1. Influence of implantation of two 15 mg. tablets of diethylstilboestrol, with or without treatment with ox pituitary extract, on the body-weight increase in normal male rats. Curve A: forty-eight control animals. Curve B: fifty-four animals treated with diethylstilboestrol. Curve C: eight oestrogenized rats treated daily with ox pituitary extract equivalent to 1·5 mg. of dried tissue, for the last 2 weeks of the experiment. Curve D: nine similar rats treated with extract equivalent to 6·25 mg. day dried tissue. Curve E: seven similar rats treated with extract equivalent to 12·50 mg. day dried tissue. Curve F: seven similar rats treated with extract equivalent to 25·00 mg. day dried tissue. Curve G: nine similar rats treated with extract equivalent to 100·00 mg. day dried tissue. Curve H: normal male rats untreated either with oestrogen or with pituitary extract.

Fig. 1 illustrates the average weights, over a period of 4 weeks, of young male rats carrying tablets of the synthetic oestrogen, compared with the results for control animals. It will be seen that, under these conditions

the normal rapid increase in body weight of the young rat was completely inhibited. When, however, other groups of rats were treated with ox anterior lobe extract for the last 2 weeks of the 4-week period of administration of oestrogen, the increases in weight illustrated in Fig. 1 were obtained. It should be explained with reference to Fig. 1 that although the pituitary-treated groups did not average precisely 114 g. in weight at the beginning of the period of treatment, nevertheless their average weights were not far from this figure, and for the purposes of comparative presentation the initial average weights have all been reduced or elevated to 114 g., with corresponding adjustment of the final weights. The figure for the 'normal' increase in weight for male rats at an average initial weight of 114 g. is based on results for animals not previously treated with oestrogen.

It will be seen from Fig. 1 that under these conditions the inhibitory influence of diethylstilboestrol on weight increase can be completely overcome by treatment with a suitable dose of ox anterior pituitary extract.

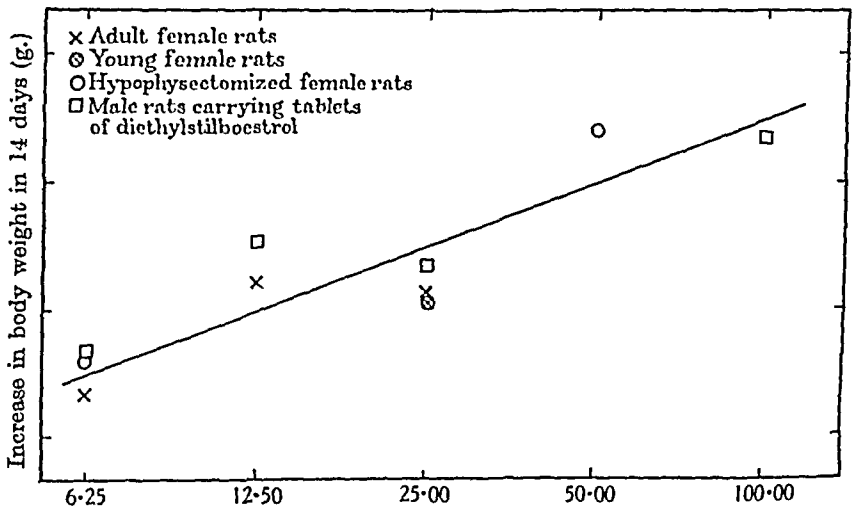


FIG. 2. Dose-response curve for different types of rat treated daily with ox anterior pituitary extract for 14 days.

Comparison of results

In Fig. 2 are plotted the results, with respect to increase in body weight, from all the experiments with pituitary extract, the scale for pituitary dosage being logarithmic. It should be pointed out that the results plotted for the hypophysectomized rat are the gross increases; the net rises in weight would be greater because of the decline in weight of the control hypophysectomized rats (Table I).

On the whole the results for the three different methods fall surprisingly close to a straight line on a logarithmic dose scale.

Increase in tail length as a criterion of growth

Normal female rats

A comparison was made of the effects of the same dose of ox, horse and sheep pituitary extracts on the increase both of body weight and of tail length. The results, which are given in Table II, show that these two data increase approximately proportionally under these conditions, the tail growth of these adult female rats being approximately 1 mm. for each 10 g. increase in body weight, whether or not they are treated with pituitary extract. Therefore the stimulus to increase in body weight arising from treatment with pituitary extract—ox, horse, or sheep pituitary —is associated in these animals with a normal rate of elongation of the tail, and it follows that the determination of tail length provides a satisfactory measure of the growth-promoting action of pituitary extracts under these conditions.

Hypophysectomized male rats

Although the hypophysectomized adult male rat tends to lose weight, yet, under the conditions of our experiments, the tails continue to elongate (Table II).

When the hypophysectomized rats were treated with ox-pituitary extract the body weights and the tail lengths increased in such a manner that the addition of each 10 g. of body weight was associated with an increase of 5·6 mm. in the tail length. The control animals, however, lost weight and gained in tail length, and when relevant corrections are made to the results for the treated animals, the net increase in tail length for each 10 g. (net) increase in body weight, is 3·2 mm., a figure within the range of these untreated, control male rats (Tables II and III).

In a footnote on p. 606 of their publication, Fraenkel-Conrat, Meamber, Simpson & Evans [1940] state that the tails of the rats of their colony continue to grow for a limited time after hypophysectomy, although their growth preparations caused approximately proportional increases in body weight and in tail length when compared with the control animals. As far as our results with hypophysectomized animals are concerned, we are in agreement with the American workers, although our observations differ in this respect from those of Freud *et al.* [1939].

Animals carrying tablets of diethylstilboestrol

Some preliminary control experiments were carried out over a period of 4 weeks to determine how far the inhibition of the normal increase in body weight, induced by the implantation of tablets of diethylstilboestrol, was associated with a diminution in the normal rate of elongation of the tail.

Table II. *Influence of anterior pituitary extracts on the body weight and tail length of groups of five to ten rats under different conditions*

Animals	Anterior pituitary extract used	Average body weight in g.			Average tail length in mm.			
		Initial	Gross increase in 14 days	Net increase in 14 days	Initial	Gross increase in 14 days	Net increase in 14 days	Gross increase per 10 g. increase in body weight
Normal female rats	None	164	12	—	165·3	1·4	—	1·2
Normal female rats	Sheep	183	23	11	175·0	2·8	1·4	1·2
Normal female rats	Horse	176	34	22	163·7	2·8	1·4	0·8
Normal female rats	Ox	182	54	42	177·4	5·6	4·2	1·0
Hypophysectomized male rats	None	131	−9	—	169·9	1·7	—	—
Hypophysectomized male rats	Ox	106	19	28	153·4	10·8	9·1	5·6 (3·2*)
Unoperated control male rats	None	162	27	—	176·1	10·7	—	3·7
Diethylstilboestrol-treated male rats	None	123	4	—	164·0	3·8	—	9·5
Diethylstilboestrol-treated male rats	Ox—6·25 mg.	133	21	17	166·2	1·3	−2·5	0·6
	Ox—25·00 mg.	129	39	35	166·1	8·4	4·6	2·2
Unoperated control male rats	None	177	22	—	177·2	7·5	—	3·4

* Net increase (mm.) in tail length per 10 g. net increase in body weight.

The results (Table III) show that despite the complete inhibition of the normal increase in body weight, the tails of the oestrogen-treated animals continued to elongate at about 40% of the normal rate. This does not agree with the observations of Garenstroom & Levie [1939] and of Levie [1939] who find that the growth of bone and the increase in tail length in the rat are arrested by a daily dose of diethylstilboestrol of 0·1 mg. or more. In our experiments the rate of absorption from the tablets averaged approximately 0·25 mg./day.

Table III. *Influence of treatment with oestrogen for 4 weeks on the body weight and tail length of male rats*

Oestrogen implanted	Total no. of animals treated	Average body weight in g.		Average tail length in mm.		
		Initial	Increase in 4 weeks	Initial	Increase in 4 weeks	Increase per 10 g. increase in body weight
Diethylstilb-oestrol	28	116	2	152·1	8·4	42·0 (?)
None	26	136	72	169·1	20·7	2·9
Oestrone*	12	98	30	146·7	15·8	5·3
None	10	97	81	147·0	32·3	4·0

* One tablet of 12 mg. implanted into each rat, of which an average of 0·04 mg./rat/day was absorbed.

That the dissociation of the rate of increase in the body weight and the rate of elongation of the tail is not a peculiarity of the action of the synthetic oestrogen, is indicated by the results, included in Table III, of an experiment with oestrone. In this instance neither the increase in body weight nor the elongation of the tail was arrested, but the rate at which weight was gained was so depressed that the rate of tail growth was 5·3 mm./10 g. increase in body weight, compared with the control value in this experiment of 4·0.

When rats carrying tablets of diethylstilboestrol were treated with a small dose of ox-pituitary extract during the second 2 weeks of the 4-week period of oestrogenization, the increase in body weight was not accompanied by a proportional increase in the rate of tail elongation (Table II). In fact the tails of one group of the pituitary-treated animals elongated more slowly than those of the control animals. When, however, a larger dose of pituitary extract was used (Table II), a substantial increase in the rate of tail growth took place, the gross rate of elongation being 2·2 mm./10 g. increase in body weight compared with the value for normal male rats of 3–4 mm.

DISCUSSION

Our results demonstrate the validity of applying the technique of tail measurement of Freud & Levie [1938] to the usual methods of growth-hormone assay. When one recalls the grossly differing proportions of the different active principles in extracts from sheep, horse and ox-pituitary glands [cf. Chance, Rowlands & Young, 1939], the relative constancy of the rate of tail growth in proportion to the increase in body weight when any of these extracts is administered (Table II) gives support to the idea that skeletal growth and increase in body weight are, in the normal rat, controlled by the same or closely related principles. This possibility is emphasized by the observation that the rate of tail growth in the untreated control adult female rats is similar, in proportion to the body-weight change, to that found in the pituitary-treated female animals (Table II). There is a substantial difference between the values for these female rats and those for the younger male control rats (Tables II and III), but such a difference does not invalidate the results for either group, provided that the data are considered in relation to those for the relevant control groups. Our results do not throw light on the question of the nature of the growth-promoting extracts on skeletal growth, but merely confirm that an apparently physiological stimulation can be obtained both in normal and in hypophysectomized rats. This aspect of the action of pituitary extracts has been investigated by Silberberg & Silberberg [1940], Ross & McLean [1940] and more recently by Ray, Evans & Becks [1941].

In the rat whose growth has been stunted by the implantation of tablets of diethylstilboestrol the relative dissociation of the rate of body-weight increase, on the one hand, and of the rate of tail elongation on the other, suggests that the oestrogen exerts an action on one of these aspects of growth more than on the other. As has been emphasized by Korenchevsky in a long series of papers [cf. Korenchevsky, Burbank & Hall, 1939] oestrogens tend to depress fat deposition and it is probable that part at least of the inhibition of the normal rate of increase in weight associated with excessive treatment with oestrogens, results from this and similar actions.

It is tempting to regard the rat whose growth has been completely prevented by excessive treatment with diethylstilboestrol as functionally hypophysectomized, and to assume that the increase in body weight that follows the administration of pituitary extract to such animals (Fig. 1) results from simple replacement therapy [cf. Richards & Kueter, 1941]. The dissociation of tail growth from body-weight increase, and the alteration of metabolic processes in such animals [cf. Janes & Nelson, 1940; Griffiths, Marks & Young, 1941; Ingle, 1941] suggest that such a simple concept

may not be correct. We do not wish to enter into an abstruse discussion of the nature of growth, but we should emphasize that, according to conditions, one can observe skeletal growth associated with a diminution in body weight (rat carrying tablets of diethylstilboestrol), or an increase in body weight associated with no increase in the rate of skeletal growth (diethylstilboestrol-treated rat receiving a small dose of pituitary extract). Which, if either, of these should be regarded as 'growth' in the general sense of the word, we do not know. We are, therefore, disinclined to recommend the use of the rat whose growth has been inhibited by implantation of tablets of diethylstilboestrol, as an object for the assay of growth-promoting preparations, however attractive are the simplicity of the technique and the relative smoothness of the dose-response curve based on body weight.

SUMMARY

The technique of Freud & Levie [1938], involving the determination of rate of tail elongation in the rat as an index of the skeletal growth, has been successfully applied to the assay of growth-promoting pituitary extracts, both in the normal female and in the hypophysectomized male rat.

When young rats, whose normal increase in body weight has been inhibited by the implantation of tablets of diethylstilboestrol, are used for the assay of growth-hormone preparations, the dose/body-weight response curve is satisfactory, but the resultant changes in body weight and tail length are not proportional.

One of us (M. G.) wishes to express his indebtedness to the Director of the National Institute for Medical Research, Sir Henry Dale, for extension of laboratory facilities. We wish to express our gratitude to Messrs Boots, Ltd., for a generous supply of 15 mg. tablets of diethylstilboestrol.

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IMPLANTATION OF SEX HORMONE TABLETS IN MAN

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Since Deanesly & Parkes [1937, 1938] first introduced the technique of subcutaneous implantation of tablets of compressed crystals of sex hormones in animals, further experiments have been conducted by those workers and by many others in different parts of the world. This method gives a continuous small absorption, simulating the normal secretion of the gonads, except that the amount absorbed is in no way controlled by any such normal factors as tissue requirements or pituitary stimuli.

Clinicians were not slow to adopt this new technique, and Bishop [1938] reported the implantation of a 14 mg. tablet of oestrone into a menopausal woman, which relieved her symptoms for about 4 weeks, after which the effect began to wane. I [Foss, 1939] reported the results of such therapy in fifteen patients suffering from kraurosis, pruritus and leukoplakia and found that, with the doses used, relief was not obtained by this means alone. The dose implanted did not exceed 60 mg. of oestrone or oestradiol.

Inadequate relief was only to be expected, as at the time the supply of tablets was limited and one's knowledge of their behaviour in the tissues was elementary. The dermatoses, such as kraurosis, leukoplakia and menopausal pruritus vulvae, are due to oestrogen deficiency, and as I [1936, 1939] had shown, very large doses of oestradiol benzoate by injection were required to improve these conditions, and the application had to be maintained at a threshold dose, which varied in each patient.

Salmon, Geist & Walter [1940] reported that 25–50 mg. of oestradiol or oestradiol benzoate caused prolonged inhibition of the pituitary gland in menopausal women as determined by the disappearance of gonadotrophin from the urine. These workers [Walter, Geist & Salmon, 1940] measured the absorption rates in women of 15–25 mg. pellets of oestradiol and oestradiol benzoate.

Loeser [1940] described a woman aged 32, who was sterile with two husbands, and she was found to have an infantile uterus less than 2 in. long measured by a probe. After giving injections of oestrogen for 6 months, with some benefit, the uterus 'reverted to its old firm consistency im-

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mediately the treatment was left off'. He then implanted five 15 mg. tablets of oestradiol to give continuous oestrogen stimulation. 249 days later, following menorrhagia, a condition of glandulocystic hyperplasia was revealed by curettage, and the uterus was found to have grown to more than 3 in. The tablets were then removed and normal menstruation was restored. Seven months later pregnancy was proved by a positive Aschheim-Zondek reaction, and at the time of the publication the patient's uterus corresponded to a fifth month pregnancy.

More reports of androgens being applied clinically by this method are available. Vest [1939] and Vest & Howard [1939] treated a series of cases of hypogonadism with considerable improvement, by the implantation of testosterone. Eidelsberg & Ornstein [1940], Spence [1940] and Moehlig [1940] have also reported satisfactory results from this method of treatment in similar cases. Loeser [1940] found this route convenient and effective for the treatment of menorrhagia by androgens, using testosterone propionate in his cases.

I carried out implantation of tablets of sex hormones first in March 1938, following Deanesly & Parkes's paper, and this is a report on the comparative absorption rates of oestrone, oestradiol, testosterone, testosterone propionate and progesterone.

In some women this method was used for the treatment of cases needing long-term therapy to avoid repeated injections [Foss, 1939], but in most of the other cases, implantations were carried out with the sole object of determining the absorption rates.

METHODS

Technique of implantation

In nearly every case the tablets were placed in the anterior abdominal wall. The skin was cleaned and swabbed with spirit and a $\frac{1}{2}$ in. incision made under local anaesthesia with 2% novocaine, containing a few drops of adrenalin.

A small lateral pocket was made in the fat to one side of the incision, the position being recorded, in each case, by a diagram on the history sheet, to facilitate later removal. Full surgical precautions were observed and the skin incision was closed by one suture, which was removed on the 7th or 8th day. The wound was covered by sterile gauze and a strip of elastoplast.

Removal

The rates of absorption obtained by Deanesly & Parkes were a useful guide to the removal of these tablets in human cases. Unfortunately, I was not able to work to a set date always, and the irregularity of attendance and other factors sometimes delayed removal. In a few cases the precipita-

tion of war prevented removal altogether, so that the tablets remained in the tissues of the patient until complete absorption.

Knowing exactly the original site of implantation marked on the skin by a very small scar, usually it was easy to find the tablets. As a routine measure a small elliptical incision was made around the initial scar under local anaesthesia with 2% novocaine, and then an area of fat, as large as necessary, was dissected out. The tablet or tablets were found imbedded in this, each separated from the other in a complete capsule of fibrous tissue, from which they were carefully dissected. They were washed very gently and dried and then reweighed. Although the tablets were smaller on removal, they maintained their shape and compactness of form. Many of the fibrous capsules were sectioned, examined and measured microscopically.

MATERIALS

The tablets of oestrone, oestradiol, testosterone propionate, testosterone and progesterone, were supplied to me ready compressed from crystals of pure hormones, and were either flat disks or cuboids. As some were hand-made by Dr A. S. Parkes, and others supplied from commercial houses, the degree of compression was probably not the same.

The tablets were weighed in dry ampoules, which were then sealed, sterilized in an autoclave, labelled and stored preparatory to use, care being taken to avoid undue shaking.

RESULTS

Summary of data

Table I gives all the relevant details. Many more tablets of oestrone and oestradiol than of testosterone propionate, testosterone or progesterone have been implanted, partly because suitable cases were available, also because these hormones were readily obtainable. The more rapid rates of absorption of testosterone propionate, testosterone and progesterone made it essential to operate again fairly soon after implantation, and this was not possible in every case. The more intense tissue reaction may have been responsible for a tendency for the tablets of the three last-named hormones to slough out, and several times this occurred, but this was not encountered when oestrone or oestradiol was used.

Forty-four separate implantations were carried out, thirty-eight of these being in women and six in men. Of these patients, eighteen had single tablets, ten had two tablets, three had three tablets, eleven had four and two had six tablets at each implantation. Nineteen consisted of implantations of oestrone, eight of oestradiol, nine of testosterone, four of testosterone propionate and four of progesterone.

Of all these series, reweighing was possible in twenty-seven cases only and Table I is compiled from these results.

Case	Age	Condition	No. of tablets	Shape	Average wt. mg.	Days implanted	Average wt. absorbed mg.	Weight absorbed %	Daily wt. absorbed mg.	Daily wt. absorbed %
(*a*) *Oestrone implantation*										
219	48	Climacteric	6	Cuboid	15·6	362	6·6	42·0	0·018	0·12
41	71	Pruritus vulvae	1	Disk	30·0	217	8·0	26·6	0·037	0·12
174	51	Leukoplakia	1	Cuboid	20·0	165	2·0	10·0	0·012	0·06
145*a*	54	,,	1	,,	21·0	151	4·5	21·5	0·03	0·14
145*b*	54	,,	2	Disk	29·5	119	4·3	14·5	0·035	0·12
222	62	,,	3	Cuboid	14·6	119	2·7	18·2	0·022	0·15
124	39	,,	1	,,	15·0	114	7·0	46·6	0·061	0·41
113	75	,,	1	,,	19·0	105	4·0	21·1	0·038	0·20
61	63	Kraurosis vulvae	2	,,	15·0	105	3·5	23·3	0·033	0·22
78	39	Galactorrhoea	1	Disk	30·0	77	2·5	8·3	0·032	0·11
127	69	Kraurosis vulvae	1	Cuboid	20·0	70	3·0	15·0	0·043	0·21
164	69	Leukoplakia	1	Disk	30·0	46	5·0	16·6	0·109	0·36
									Mean	**0·18**
(*b*) *Oestradiol implantation*										
127	69	Kraurosis	3	Cuboid	18·3	196	10·0	54·5	0·05	0·28
90	60	Atrophic vaginitis	1	,,	14·0	147	6·4	45·7	0·044	0·31
142	56	Leukoplakia	2	,,	13·8	115	6·3	45·4	0·054	0·40
227	53	Kraurosis	1	,,	14·0	112	6·0	42·9	0·050	0·36
296	67	Leukoplakia	4	,,	16·0	98	4·4	27·3	0·044	0·28
90	60	Atrophic vaginitis	4	,,	15·4	66	6·8	44·4	0·103	0·67
222	62	Leukoplakia	4	,,	15·2	35	2·2	14·5	0·063	0·41
									Mean	**0·38**
(*c*) *Testosterone propionate implantation*										
161	34	Radium menopause	1	Disk (thick)	190	21	37	19·5	1·76	0·92
392	32	Impotence	1	,,	186	11	16	8·6	1·45	0·78
									Mean	**0·85**
(*d*) *Testosterone implantation*										
168	40	Metropathia haemorrhagica	4	Disk	50·5	60	34	67·3	0·56	1·12
78	42	Galactorrhoea	4	,,	48·8	42	19·9	40·8	0·47	0·97
C	82	Male senility	4	,,	48·8	36	25·8	52·8	0·71	1·46
									Mean	**1·18**
(*e*) *Progesterone implantation*										
61*a*	63	Kraurosis	1	,,	25·0	49	18·0	72·0	0·37	1·47
61*b*	63	,,	1	,,	25·0	31	9·0	36·0	0·29	1·16

In the remaining seventeen cases, removal was attempted in six, but by that time absorption was complete and tablets could not be found. This group consisted of one case implanted with oestradiol after 224 days, three cases with testosterone after 195, 195 and 185 days, and two cases with testosterone propionate after 28 and 39 days.

A small number of tablets sloughed out, and this group consisted of two cases with testosterone and two cases with progesterone tablets.

A few patients had tablets left in their tissues at the outbreak of war, as I had intended to investigate the absorption over a long period—thus five cases had one or more tablets of oestrone still imbedded after periods of 415, 415, 412, 380, and 254 days. A eunuch had four tablets of testosterone in his tissues which had been implanted 78 days previously.

Absorption rates

The average percentage weight absorbed per day in the oestrone series was 0·18, with a range of 0·06–0·41, whilst the average percentage weight absorbed per day in the oestradiol series was 0·38, with a range of 0·28–0·67. Assuming a constant rate of absorption, such tablets of oestrone would last 550 days and oestradiol only 260 days.

From the small number of cases of androgen implantation available, it is suggestive that testosterone propionate is less rapidly absorbed than testosterone, but twice in one patient I was unable to find four tablets of testosterone propionate after 28 and 39 days, but as this patient was excessively obese with literally about 3 in. of fat over the abdomen, it is conceivable that I may have lost them and they may not have been absorbed.

My figures show that testosterone propionate tablets lose 0·85 % of their weight per day and so have a calculated life of 118 days, whereas testosterone loses 1·18 % per day and would last 85 days if steadily absorbed.

Progesterone tablets were not readily obtained in sufficient number or size, and only twice was it possible to remove or reweigh them. The percentage weight lost per day averaged 1·31, which gives a duration of approximately 76 days.

Factors influencing absorption

Deanesly & Parkes suggested that the rate of absorption must depend largely on the surface area of the tablets implanted and will, therefore, be absolutely greater though proportionately less from a large tablet than a small one, and as absorption proceeds and the tablets become smaller, the absolute amount absorbed per month and also the percentage in terms of the original weight of the tablet will become less. The mean values of percentage loss of weight per day give a calculated life of about 550 days for oestrone, 260 days for oestradiol, 118 days for testosterone propionate,

85 days for testosterone and 76 days for progesterone. If one takes the mean daily percentage weight absorbed in five cases where oestrone tablets remained implanted for between 119 and 362 days, the figure is 0·11, whereas in another five cases of implantation for 46–105 days the figure is doubled (i.e. 0·22 %). This bears out the animal experimental results of Parkes & Deanesly, showing that absorption is more rapid at the beginning and slows down as the tablet becomes smaller (see Fig. 1). Exactly the same is seen in the oestradiol series; in three cases tablets implanted 115–196 days have a mean daily percentage loss of 0·33, whereas in three other cases tablets implanted for a shorter time (35–98 days) lost 0·44 % daily.

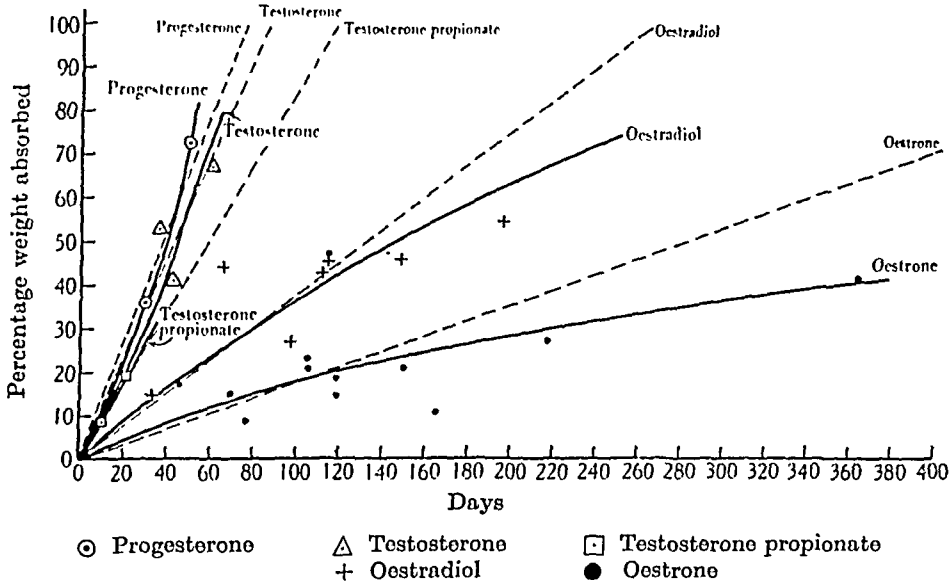


Fig. 1. Absorption of sex hormones from tablets implanted in man. - - - - Mean percentage weight absorbed daily assuming steady absorption rate. —— Actual results showing reduction in absorption rate of oestrone and oestradiol with time.

Therefore, it is suggestive that unless definite pitting of the surface of tablets occurs, thereby increasing the surface area, the length of life of hormone tablets calculated from the mean figures of the whole series is on the short side, and that, in fact, oestrone may last 700–800 days, oestradiol about 360 days, testosterone propionate 140 days, and testosterone and progesterone about 100 and 80 days respectively.

The absorption rate may depend on several factors:

(1) Size and shape of tablets.
(2) The degree of compression of the tablets.
(3) The relative solubility of the hormone in lipoids.
(4) The degree of tissue reaction around the tablets.

In a recent paper Emmens [1941] has pointed out that whatever the mechanism of absorption, the surface area of the tablets will, other conditions being equal, determine the rate of absorption. The initial absorption rate theoretically should be proportional to $\frac{2}{3}W$, where W is the weight, but subsequent absorption will depend on the actual shape of the tablet and its behaviour in the tissues. A tablet which is compressed loosely may become eroded and pitted, increasing its surface area and so hastening the absorption as time goes on, although its weight is becoming less. In this series of experiments tablets were supplied from several different sources, and it is likely that their compactness differed considerably, and after removal it was noticed that pitting was more evident in some than in others.

I have been unable to obtain any facts about the relative solubilities of the sex steroids used, in body fat or other lipoids, but it is likely that this factor is of great importance. The other factor which must show some individuality in man is the degree of tissue reaction around the tablets, and this also may in turn be dependent on the solubility of the particular hormone in body fats; there is very little reaction around sterilized tablets of sex hormones, but they are all treated in much the same way as a foreign body, with the production of granulation tissue, giant cell formation and formation of a fibrous tissue barrier. This gives rise to the capsule, which is invariably found around each tablet on removal. Sometimes they are covered immediately with a very thin layer of hormone mush which is in direct contact with the capsule wall.

In many cases I have carefully dissected out these capsules, which have been sectioned and measured (Table II). Oestrone capsules are thin and show little granulation tissue, whereas progesterone, testosterone and testosterone propionate capsules are thicker and more fleshy, showing more granulation tissue and giant cells. Oestradiol fills an intermediate position between them.

This then shows a similar gradation to the absorption rates of these hormones and it is reasonable to connect the two observations with each other.

It is not possible to say whether the more intense tissue reaction and phagocytosis account for the more rapid absorption, or whether this is secondary to a greater solubility of the more rapidly absorbed hormones, or whether it is due to other causes unknown. The fact remains that the tissue reaction around the more rapidly absorbed hormones is more active. Geist, Walter & Salmon [1940] suggest that the capsules might have a marked retarding effect on the rate of absorption of the hormone and they connect the progressively diminished absorption rate of a series of ten oestradiol tablets with the growing thickness of the capsule around the pellet. Adequate figures of results were not given in their paper for com-

Table II. *Thickness of capsules and percentage weight absorbed per day*

Case no.	Thickness of capsule μ	Days implanted	% weight absorbed per day
(*a*) *Oestrone*			
127	62	70	0·21
78	28	77	0·11
124	23	114	0·41
113	56	105	0·20
145*a*	275	151	0·14
145*b*	87	119	0·12
174	125	165	0·06
41	20	217	0·12
145*c*	75	119	0·12
Mean	**76**		**0·145**
(*b*) *Oestradiol*			
227	87	112	0·36
142	50	115	0·40
127	125	196	0·28
222	165	35	0·41
Mean	**109**		**0·36**
(*c*) *Testosterone*			
78	325	42	0·97
168	375	60	1·12
Mean	**348**		**1·05**
(*d*) *Progesterone*			
61	287	31	1·16

parison, but in my cases it was noticeable that tablets of progesterone, testosterone and testosterone propionate disappeared more quickly and had a much thicker capsule than the slowly absorbed oestrone tablets; the latter had a very thin capsule whether they were removed after 70 days or after 217 days, whereas the tablets of progesterone and testosterone have thicker, more vascular capsules with many giant cells and small round cells.

It appears that owing to factors as yet unexplained, the reaction around oestrone tablets is generally slight. The tablets are then isolated by fibrous tissue, which may account for the reduction in absorption rate.

Forbes [1941] showed that in animals, where a tablet was surrounded by a thicker, more atypical capsule than usual, the rate of absorption appeared to be most often greater than usual. In my cases all the more rapidly absorbed tablets, *i.e.* progesterone, testosterone and testosterone propionate, had a more vascular and thicker capsule.

A careful investigation into the amount of fibrous tissue around the tablets is required, because this rather than the actual thickness of the

capsule would seem to be an important factor in reducing effective absorption.

In one case a tablet of oestrone was removed (from patient 164) after implantation for 46 days in which time it had lost 5 mg. = $16\frac{2}{3}$% of its weight. It was washed, dried and sterilized in the same way as all the others of the series and then was re-implanted in a different patient (No. 9)—after 147 days the tablet was removed and was found to have lost no more weight. This was the only tablet which was used more than once.

Clinical effects

Oestrone and oestradiol tablets were implanted in women for chronic conditions needing continued oestrogen therapy after preliminary high dosage by injection, for example leukoplakia and kraurosis vulvae and menopausal pruritus vulvae, climacteric symptoms and galactorrhoea. In addition, most of the cases of vulval irritation were given an ointment of oestradiol containing 2·5 mg. in 25 g. of a non-irritant base. Comfort by this therapy was maintained in only a small number of these patients for a few months after implantation. But there is evidence that some of them had sufficient hormone in the circulation, as flushes were controlled. Case 219, a woman aged 48, who had had infrequent scanty menstruation for some years, started uterine bleeding 88 days after implantation of oestrone and this lasted quite copiously for 11 days. After a further 63 days she bled again for 9 days, and although the tablets were not removed for 362 days, she had no more bleeding. In her case 94 mg. had been implanted in four tablets, and the calculated daily dose absorbed in this time was 0·108 mg. of oestrone. There was no effect on the case of galactorrhoea, using implanted oestrogens.

Adequate control of such cases is greatly facilitated by taking periodic vaginal smears, and if the type of maintenance dose of oestrogen by injection or by mouth is known, an approximate calculation of the amount of oestrone to be implanted can be estimated.

Tablets of testosterone were implanted in four women and four men—nine separate implantations being carried out altogether. This method of application was used as injections of male hormone had been given previously for various reasons for a long time. In five cases there was no clinical effect, but in one woman with galactorrhoea (case 78), previously treated unsuccessfully with oestrogen, the persistent flow of milk was temporarily stemmed by 195 mg. A eunuch [Foss, 1937], after a relapse due to cessation of therapy, once more regained full sexual function 3 days after implantation of 307 mg. of testosterone. Erection, orgasm and even pollution occurred for a period of about 3 weeks, after which the effect began to wane.

Testosterone propionate tablets were implanted in one man with impotence and in three women. The man showed no improvement with a dose of 186 mg. in one tablet. A woman with galactorrhoea previously implanted with testosterone, after two implantations of 49 and 47 mg. of testosterone propionate remained free from milk secretion for 4 months, and the tablets were not removed. Another woman, case 161, following a radium menopause, was relieved of her flushes.

As a practical therapeutic measure tablet implantation of sex hormones can be used for long-term therapy where injections, percutaneous application or oral therapy are either impossible, undesirable or impracticable. Bearing in mind the variables which alter the absorption rate, it is probably best to implant a large round tablet of oestrone or testosterone propionate, where a small dose for a long time is required. Many large flat disks of oestradiol or testosterone would maintain a higher level of dosage for a shorter time.

Emmens [1941] has shown that esterification decreases the rate of absorption, and thus skilful use of one of the many esters available should greatly facilitate the task of the clinician in choosing the type of tablet suitable for implantation in an individual condition, in order to give a required dose.

SUMMARY

Weighed tablets, made from compressed crystals of oestrone, oestradiol, testosterone propionate, testosterone or progesterone, were implanted in male and female patients subcutaneously for varying periods of time, then removed and reweighed. From these figures the mean rates of absorption have been found to increase in the above order. Some individual cases are described briefly. The tissue reaction of these hormones was found to vary directly with the rate of absorption.

It is suggested that this method is useful where continued therapy with the sex hormones in small doses is required. It may be combined with occasional 'boosting' doses by injection, inunction or by mouth.

The hormone tablets used in this work were kindly provided by Dr A. S. Parkes, by Organon Laboratories, and by Messrs Schering and Ciba, Ltd.

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SEROLOGICAL PROTECTION AGAINST THE DIABETOGENIC SUBSTANCE OF THE ANTERIOR PITUITARY GLAND

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A number of investigators have observed that, when dogs are treated with diabetogenic anterior pituitary extract, the symptoms of diabetes may make only a transitory appearance despite the daily administration of very large amounts of extract. Young [1937] found that the diabetic condition could be induced to reappear when the amount of extract injected daily was suitably increased, and did not believe that the refractory state resulted from the appearance in the blood of antihormones. However, Dohan & Lukens [1939] found that when a dog had been treated with daily injections of crude anterior pituitary extract for over 9 months, its serum possessed anti-diabetogenic activity. Administration of such serum to completely depancreatized dogs induced a marked decrease in the intensity of glycosuria. Young [1938], on the other hand, using preparations of prolactin with marked glycotropic (anti-insulin) activity, was able to produce sera with marked anti-prolactin action but with no detectable anti-glycotropic activity.

Experimental results suggesting the existence of anti-diabetogenic activity in sera raised against pituitary extracts were obtained by us early in 1939. Our investigations have subsequently been extended to include experiments on the absorption of antidiabetogenic sera with various pituitary fractions.

METHODS

For the production of serum English hutch rabbits bred at the Institute were used and subjected twice a week to intravenous injections of a partially fractionated[2] extract of the anterior glands. The amount of extract injected was equivalent to 4 g. of gland per week, and the injections were continued for from 8 to 12 weeks, when the antibody content of the serum was thought to be sufficiently high.

[1] Working under a full-time grant from the National Health and Medical Research Council, Australia.

[2] The euglobulin fraction was removed by one-third saturation with ammonium sulphate.

The potency of this serum is difficult to evaluate, as the antigen on which it is supposed to act is unknown. It was first absorbed with ox serum in order to remove the unspecific antibodies against ox serum proteins and then tested against a prolactin fraction and the 'D' fraction of Chance, Rowlands & Young [1939].

In practice, strong complement fixation was obtained in a dilution of 1/1600 and 1/3200 in the case of the 'D' and prolactin fractions respectively. The serum, therefore, contains antibodies against both the alcohol-ether precipitable anterior pituitary proteins and those whose isoelectric points lie between *p*H 4·5 and 6·5.

Partially depancreatized male hooded rats were used as test animals. Following recovery from operation they were transferred to metabolism cages and fed on a diet of bran, casein and dried carrot in the proportions of 1 : 2 : 1 respectively. Water was given *ad lib.*

After a few days control period, the animals were given two intraperitoneal injections (each equivalent to 1·5 g. fresh gland) per day of pituitary extract.

RESULTS

As Table I shows, the injection of anterior pituitary extract (A.P.E.) is followed by marked increase in the urinary excretion of glucose, which is maintained in the case of the control animal (rat 1).

Table I. *Effect of daily* A.P.E. *injections on glucose excretion of partially depancreatized rats*

	24 hr. excretion of glucose in mg.				
Rat no.	1st day	2nd day	3rd day	4th day	5th day
(a) A.P.E. *injections*					
1	0·0	0·0	50·3	49·8	—
(b) A.P.E. *injections + injection of immune serum on 2nd day*					
2	9·2	29·5	7·9	Trace	—
3	5·7	36·8	0·0	0·0	—
(c) A.P.E. *injections + injection of normal serum on 2nd day*					
12	5·0	38·7	67·0	80·4	92·3
13	20·0	41·6	64·0	76·3	97·5

Rats 2 and 3 received, in addition to the anterior pituitary extract, an injection of 3 ml. of immune serum after which the urinary glucose disappeared. Thus, the serum has not only protected against the diabetogenic effect of the anterior pituitary extract, but has also abolished the glycosuria arising from the loss of pancreatic tissue. The administration of normal rabbit serum does not result in any protection, as shown in rats 12 and 13.

It is of further interest to ascertain the nature of the diabetogenic principle in so far as its antigenic properties are concerned, and with this

end in view the rabbit anti-serum was absorbed with the prolactin, growth and thyrotrophic principles.

The animals taken for the experiment, the results of which are shown in Table II, were given two daily injections of anterior pituitary extract (equivalent to 3 g. gland), and in addition 3 ml. of immune serum on the third day of the experiment.

Table II. *Effect of absorbing immune serum with various pituitary fractions*

Rat no.	Total urinary glucose (mg.) 1st day	2nd day A.P.E.	3rd day A.P.E. and serum	4th day A.P.E.	5th day	Serum absorbed with
4	12·0	13·0	32·0	71·0	73·0	Prolactin* factor
5	14·0	20·0	49·0	59·0	70·0	
6	15·0	16·0	46·0	24·0	25·0	Growth factor
7	16·0	16·0	43·0	< 6·0	10·0	
8	22·0	24·0	35·0	6·0	11·0	Thyrotrophic factor
9	10·0	18·0	25·0	13·0	13·0	
10	18·0	22·0	32·0	49·0	Traces	Untreated
11	21·0	19·0	56·0	5·0	Traces	

* Containing both adrenotrophic and mammotrophic factors.

Protection against the diabetogenic action of anterior pituitary extract is afforded to animals 6–11 inclusive. In animals 10 and 11 treated with unabsorbed serum there is also a protection, and, further in accord with the behaviour of animals 2 and 3, Table I, the glycosuria due to partial pancreatectomy has disappeared. In contrast to this, whilst there is a protection against the action of the diabetogenic principle, the initial glycosuria is not abolished in animals 6–7 and 8–9, which were treated with serum absorbed with growth and thyrotrophic factors respectively. This is almost certainly due to the partial removal of protective antibodies by the relatively impure growth and thyrotrophic fractions. The most likely impurities would be traces of prolactin, but the possibility of a further contaminant not identical with the known fractions of the anterior pituitary gland must be kept in mind.

Contrary to these results, serum which has been absorbed with prolactin fractions gives no protection against the diabetogenic effect of the extracts. It can, therefore, be stated that the diabetogenic and prolactin fractions of the anterior pituitary gland are similar as regards their antigenicity, but that there is probably no serologic association between these fractions and the growth and thyrotrophic principles.

This paper constitutes a preliminary report of the work, the full details of which will be published later.

SUMMARY

1. The serum of rabbits treated with an anterior pituitary extract abolished the glycosuria in partially depancreatized rats and inhibited the diabetogenic action of pituitary extracts in the same animals.

2. The antidiabetogenic activity of the serum was absorbed by growth and thyrotrophic pituitary extracts but not by a prolactin fraction; the activity abolishing the normal glycosuria in partially depancreatized rats was absorbed by all three extracts.

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EFFECT OF HORMONES ON DEGENERATION OF THE *X*-ZONE IN THE MOUSE ADRENAL

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Between the 'permanent' cortex and medulla of the mouse adrenal there is a transitory cortex variously called the *X*, interlocking, boundary, or androgenic zone. At the height of its development the zone occupies about half the total width of the cortex. Its development, histology and variations in intact animals have been described elsewhere [Masui & Tamura, 1926; Tamura, 1926; Howard, 1927, 1930, 1937; Deanesly, 1928, 1938; Whitehead, 1933; Waring, 1935; Waring & Scott, 1937].

Sections of glands from a mouse 4 weeks old, of either sex, show a well-defined *X*-zone of highly eosinophil cells interlocking with the medulla. In the intact male, rapid degeneration of the zone begins at 28–30 days and leaves a connective tissue band between the medulla and permanent cortex.

In males castrated before puberty, the zone does not degenerate at the usual time but increases in size. Degeneration does eventually occur in old castrated males. Injection of androgens evokes rapid degeneration in the castrated male [Martin, 1930; Poll, 1933; Deanesly & Parkes, 1937; Starkey & Schmidt, 1938; Howard, 1940].

The *X*-zone of virgin females also degenerates, leaving a fibrous capsule to the medulla. In this sex the process is more protracted. In some strains degeneration may commence at 30 days [Howard, 1927], but in the mice used in the present experiments the zone is still intact until at least 70 days.

Pregnancy at any age precipitates rapid degeneration of the zone. Five days may suffice for its complete degeneration, and it has always reached this condition by the twelfth day [Howard, 1927; Deanesly, 1928; Takewaki, 1936]. According to these authors degeneration does not occur during pseudopregnancy nor does trauma of the pseudopregnant uterus evoke degeneration [Takewaki]. Since implantation of the embryo occurs at the sixth day it is evident that placentation is not essential to the degenerative process.

Implantation and injection of anterior lobe substance and the injection of chorionic gonadotropin evoke degeneration of the zone in intact but not spayed females (*see* summary of literature by Takewaki [1935]).

Various theories of the physiological role of the *X*-zone have been discussed by Howard [1927, 1930, 1937, 1939*a*, *b*, 1940], Howard & Grengadom [1940], Grollman [1936], Leblond & Gardiner [1938] and Gersh & Grollman [1939].

The present paper describes experiments designed to elucidate the physiological basis of degeneration during pregnancy. This object was not achieved, but suggestive results were obtained.

MATERIAL AND METHODS

The mice were albinos taken from a colony built up from half a dozen given by Dr Parkes in 1933. The sexual characteristics of the parent stock have been described by Parkes [1925, 1927] and the histology of the adrenals by Deanesly [1928].

Males were castrated at 23–25 days through a single abdominal incision. Females were spayed through two small lumbar incisions.

In most experiments the left adrenal was removed prior to injections. It was fixed in Bouin's fluid, sectioned in paraffin and stained with haematoxylin and eosin. Sections to confirm the presence of fibrous tissue were stained with Mallory. At the end of the injections the second adrenal was treated similarly. For the present purpose there is no objection to this method of control. Scott (unpublished) in a study of 750 glands found no difference between the *X*-zone of the right and left glands. Whitehead [1933] observed no changes in the remaining gland after unilateral adrenalectomy. In some experiments animals were used without first removing one adrenal, reliance being placed on the known age relationships of the zone as control.

All aqueous solutions were administered twice, oily solutions once, daily.

THE EFFECT OF INJECTING VARIOUS HORMONES

Effect of oestrone injections

Previous work indicates that the response evoked by oestrogens both in the *X*-zone and permanent cortex varies greatly with the dose [Martin, 1930; Burrows, 1936; Cramer & Horning, 1937; Deanesly & Parkes, 1937; Lacassagne & Raynaud, 1937; Deanesly, 1939].

Marrian & Parkes [1930] found that 200 m.u. of oestrogen were necessary to evoke full oestrous symptoms in a spayed mouse. The amount of oestrone equivalent to 1 m.u. varies greatly according to different workers, but according to Burn [1937] Marrian gives 0·06 μg. of oestrone as 1 m.u. One international unit is the oestrus-producing activity of 0·1 μg. oestrone. So 10–20 μg. (0·01–0·02 mg.) of oestrone have similar oestrogenic activity to the 200 m.u. injected by Marrian & Parkes. There are no data for the amount of oestrogens in the mouse body during pregnancy. At this time the oestrogen content of human serum increases considerably. So larger quantities than the minimum (i.e. 0·01–0·02 mg.) necessary to produce full oestrous symptoms were injected into some animals. Table 1 records the results obtained

Table 1. *Effect of daily injections of oestrone in intact and ovariectomized female mice*

No. of animal	Age at start of experiment in days	Oestrone treatment: Total dose mg.	Oestrone treatment: Period days	Histological condition: 1st adrenal	Histological condition: 2nd adrenal
(*a*) *Intact*					
1	40	0·01	14	—	*X*-zone intact
2	40	0·035	4	—	*X*-zone intact except for incipient degeneration in places
(*b*) *Spayed and one adrenal removed prior to treatment*					
3	60	0·075	5	*X*-zone intact	As no. 2
4	60	0·15	5	,,	*X*-zone haemorrhagic with clear evidence of disintegration
5	60	0·305	10	,,	*X*-zone large and intact

when varying amounts of oestrone were injected into intact and ovariectomized female mice. It will be seen that the treatment caused no degeneration of the *X*-zone.

Autopsy showed that all the oil was not absorbed in animals receiving the larger injections, but the vaginae of these animals were more heavily cornified than during normal oestrus.

Since degeneration of the *X*-zone can take place within 5 days during a normal pregnancy and large doses of oestrone over longer periods did not consistently lead to its destruction, we may conclude that degeneration during pregnancy is not due to an increased concentration of oestrogen in the circulation.

Effect of progesterone injections

Early experiments made by the writer in 1933, like the similar ones of Martin [1930], gave negative results. They were not published. In that series an oily extract of corpus luteum, marketed by Parke, Davis and Co., was injected for periods up to 16 days. No gland showed definite signs of *X*-zone degeneration. In my 1938 experiments synthetic progesterone replaced the oily extract. Robson [1938*a*] showed that pregnancy can be maintained in spayed mice by daily injection of 1 mg. of progesterone. His later studies [1938*b*] showed that 1·5 mg. daily reproduced more faithfully the effects of the amount of progesterone-like substances present during normal pregnancy. He reached this conclusion from the following observations: (i) more oestrogen is necessary to cause vaginal cornification in pregnant animals than in non-pregnant spayed animals [Parkes & Bellerby, 1926]; (ii) 'inhibition of the vaginal action of oestradiol during pregnancy is similar to that produced in non-pregnant spayed animals by the administration of about 1·5 mg. of progesterone per day' [Robson, 1938*b*].

Using these data as a guide the experiments recorded in Table 2 were made. Table 2 shows that doses of progesterone that might be considered of physiological

Table 2. *Effect of daily injections of progesterone into 40-day-old intact or ovariectomized female mice. Autopsy 1 day after last injection*

No. of animal	Progesterone treatment: Total dose mg.	Progesterone treatment: Period days	Histological condition after experiment
(*a*) *Intact*			
1	7·0	7	*X*-zone intact
2	7·0	7	*X*-zone intact but thin connective tissue band forming between it and medulla
3	10·5	7	*X*-zone intact
4	10·5	7	As no. 2, but connective tissue not so well marked
5	10·5	7	*X*-zone intact
6	18·0	9	*X*-zone intact
(*b*) *Spayed one week previously*			
7	10·5	7	*X*-zone intact
8	10·5	7	*X*-zone intact
9	18·0	9	*X*-zone intact

significance do not evoke degeneration of the *X*-zone. Howard & Grengadom [1940] reached the same conclusion after injecting larger doses and implanting tablets of progesterone

Effect of progesterone injections after oestrone

Progesterone produces its typical effect on the uterine endometrium of rabbits only when injected into intact animals in oestrus or into castrated animals previously injected with oestrogens. On the assumption that *X*-zone behaviour might prove analogous, mice were first injected with oestrone and then with progesterone. This procedure was tentatively tried out on nos. 1 and 2 of Table 2, by starting the progesterone injections at oestrus. There are no data on the amount of oestrogens present during early pregnancy, so small doses were used in the first experiments and the amounts increased in later ones.

The results are summarized in Table 3. They provide no reason for believing that oestrone and progesterone in combination are responsible for *X*-zone degeneration during pregnancy.

Table 3. *Effect of daily injections of oestrone and progesterone into intact or ovariectomized female mice*

No. of animal	Age at start of experiment days	Oestrone treatment* Total dose mg.	Oestrone treatment* Period days	Progesterone treatment* Total dose mg.	Progesterone treatment* Period days	Histological condition of adrenal after injection
(*a*) *Intact*						
1	40	0·07	7	7·0	7	*X*-zone intact
2	40	0·02	2	10·5	7	,,
3	40	0·02	2	10·5	7	,,
4	40	0·035	4	10·5	7	,,
5	40	0·02	2	18·0	9	,,
6	40	0·035	4	18·0	9	,,
(*b*) *Spayed and one adrenal removed prior to treatment*						
7	45	0·065	4	12·0	8	*X*-zone intact
8	60	0·305	10	9·0	6	*X*-zone intact. Marked vacuolization of zona reticularis
9	60	0·185	12	12·0	8	,, ,,

* Except for nos. 8 and 9, oestrone was injected on successive days, followed by daily injection of progesterone. Nos. 8 and 9 received oestrone alone for 4 days and both oestrone and progesterone for the remaining days. All animals autopsied 1 day after last injection.

Effect of injections of oestrone together with chorionic gonadotropin

It seems that degeneration of the *X*-zone in pregnancy is not due to oestrone or to progesterone alone or in combination. Injection of gonadotropins into intact but not spayed females evokes degeneration. Thus the ovary is in some way involved. Takewaki believed that 'the participation of a third ovarian hormone...may be possible' [Takewaki, 1935]. Starkey & Schmidt [1938] suggested that an ovarian androgen is responsible. Another interpretation may be put forward which has not been considered hitherto. Takewaki observed that the *X*-zone may degenerate in intact females injected with gonadotropins while the vagina is still showing an oestrous smear. This is open to several interpretations. One is that oestrogens and gonadotropin act synergically to evoke degeneration of the zone. During the course of

[1] Since these experiments were performed it has been shown that mouse uteri react to progesterone without previous sensitization with oestrogens [Howard & Grongadom, 1940].

preliminary experiments to confirm that gonadotropins do not affect the *X*-zone in gonadectomized animals, degeneration did occur in some castrated males injected with a crude extract of pregnancy urine. The extract exhibited oestrogenic activity. Androgenic properties were not tested for, so no definite conclusion could be drawn. The possibility of simultaneous or synergic action of oestrone and chorionic gonadotropin evoking a response similar to that of androgens was deemed of sufficient importance to justify further test using a purified gonadotropin. So gonadectomized males and females were injected with various doses of oestrone and gonadotropin. Two procedures were employed: (*a*) oestrone and gonadotropin were injected simultaneously. Twelve virgin females were spayed at 30 days and one adrenal was removed at 42 days. Injections started at 44 days and finished at 53 days. Autopsy was performed at 54 days. All animals received 30 i.u. chorionic gonadotropin ('Pregnyl') per day, four received 0·005 mg. of oestrone per day and four received 0·01 mg. per day. The *X*-zone was intact in all adrenals at the end of treatment. (*b*) Gonadotropin was injected daily into castrates previously treated with oestrone. Table 4 shows that this treatment brings about degeneration of the *X*-zone. Similar quantities of oestrone alone or of gonadotropin alone do not have this effect.

Table 4. *Effect of daily injections of oestrone followed by daily injections of chorionic gonadotropin into castrated male mice*

No. of animal	Age at start of injections† days	Chorionic gonadotropin* injections: Daily dose i.u.	Chorionic gonadotropin* injections: Period (days inclusive)†	Killed	Histological condition of second adrenal
1	50	30	6–12	13th day	*X*-zone crushed and degenerating
2	70	30	6–12	,,	,, ,,
3	50	30	6–15	17th day	,, ,,
4	70	30	6–15	,,	,, ,,
5	50	90	6–12	13th day	,, ,,
6	70	90	6–12	,,	,, ,,
7	50	90	6–12	Died on 12th day	,, ,,
8	70	90	6–15	17th day	*X*-zone slightly reduced. No degenerative changes obvious
9	70	None		13th day	*X*-zone not reduced. Completely intact

* Pregnyl (Organon, Ltd.).

† Nine males castrated at 25 days. All received 0·02 mg. oestrone in oil per day for 3 days (i.e. total of 0·06 mg.). On 5th day first adrenal removed. All these showed well-developed intact *X*-zone.

Effect of pituitary gland implantations

To supplement these observations on the effect of chorionic gonadotropin after oestrone, pituitary implants were made into gonadectomized animals after injections of oestrone. The controls received implants but no oestrone. Pituitaries were removed from rats and inserted subcutaneously. The two lobes of the pituitary were not separated because it was desired to make the implantations rapidly and it was not expected that posterior lobe autacoids would exert any influence.

Table 5 summarizes the experiments. The results were unexpected. The *X*-zone was degenerating, or had degenerated completely, in all gonadectomized animals that received only pituitary implants (nos. 1–5, Table 5). Since implants of anterior

Table 5. *Effect of implanting adult female rat pituitaries into spayed female and castrated male mice*

No. of animal	Sex	Treatment	Histological condition: 1st adrenal	Histological condition: 2nd adrenal
1	Female	Spayed and one adrenal removed 47 days, two pituitaries implanted 48 days and two at 52 days. Killed 54 days	*X*-zone intact	*X*-zone degenerated
2*	Female	Spayed 66 days old. One adrenal removed and two pituitaries implanted at 74 days. Two pituitaries implanted 78 days. Killed 81 days	*X*-zone large and intact	*X*-zone degenerating
3*	Female	As no. 2	As no. 2	*X*-zone degenerated
4*	Female	As no. 2 but 1st adrenal removed and two pituitaries implanted 66 days. Two pituitaries implanted 70 days. Killed 73 days	As no. 2	*X*-zone degenerating
5*	Female	As no. 4	As no. 2	*X*-zone degenerated
6	Female	Spayed 26 days. 0·05 mg. of oestrone injected between 31 and 41 days. 1st adrenal removed at end of oestrone treatment and two pituitaries implanted same day. Two pituitaries implanted 45 days. Killed 49 days	*X*-zone intact	*X*-zone intact
7	Male	Castrated 21 days. 0·05 mg. of oestrone injected 26–31 days. 1st adrenal removed at end of oestrone treatment and two pituitaries implanted same day. Two pituitaries implanted 35 days. Killed 39 days	*X*-zone intact	*X*-zone intact

* Animals from a different albino stock in which a large *X*-zone is developed which does not degenerate until late in life.

lobe substance do not evoke *X*-zone degeneration in castrates [Takewaki, 1935], the degeneration in these animals must be attributed to the presence of posterior lobe tissue in the implants used in the present experiments. The two animals (nos. 6 and 7) that were injected with oestrone prior to implantation of pituitaries had intact *X*-zones at the end of the experiments. So it seems that oestrone antagonizes the destructive effect of the posterior lobe on the *X*-zone.

An attempt was made to confirm these findings with injections of posterior lobe extracts uncontaminated with anterior lobe substance (Table 6). Unfractionated extracts of posterior lobe pituitary exhibit pressor, antidiuretic, melanophore-expanding and oxytocic properties. Two fractionated extracts were injected: (i) Pitressin (Parke, Davis and Co.) which contains the first three properties mentioned with only a trace of oxytocin, (ii) a preparation of melanophore-expanding hormone uncontaminated with other posterior lobe principles [Landgrebe & Waring, 1941]. The amount of the latter injected contained roughly the same quantity of melanophore excitant as 0·5 ml. of Pitressin. The experiments made, though small in number, support the view that a posterior lobe hormone (probably vasopressin) has a destructive effect on the *X*-zone and that oestrone inhibits this effect.

The larger doses of Pitressin injected (nos. 1 and 2, Table 6) caused discoloration of the kidneys. So the experiments were discontinued as being unlikely to furnish data of physiological significance.

Table 6. *Effect of injecting posterior lobe hormones into male and female mice*

No. of animal	Sex	Treatment	Histological condition: 1st adrenal	Histological condition: 2nd adrenal
1	Male	Castrated 25 days. 1st adrenal removed 70 days. 0·5 ml. of Pitressin injected 71–73 days inclusive. Killed 74th day	*X*-zone intact	*X*-zone degenerated
2	Female	Intact. 1st adrenal removed 50 days. 0·5 ml. of Pitressin injected each day 53–57 days inclusive. Killed 58th day	*X*-zone intact	*X*-zone intact except for a few vacuoles
3	Female	As no. 2 but injected 0·1 ml. of Pitressin per day	*X*-zone intact	*X*-zone intact
4	Female	,, ,,	*X*-zone intact	*X*-zone degenerating
5	Female	As no. 2 but injected 0·5 ml. per day of solution of L.W. melanophore-expanding hormone (0·4 mg./ml.)	*X*-zone intact	*X*-zone intact
6	Female	,, ,,	*X*-zone intact	*X*-zone intact

DISCUSSION

The physiological basis of *X*-zone degeneration during pregnancy is not yet clear. Leblond & Nelson [1937] showed that the *X*-zone is only found in mice with an intact pituitary. Hypophysectomy also reduces the permanent cortex but does not obliterate it. So it is evident that in the intact animal both the permanent cortex and the *X*-zone are maintained by pituitary secretions. There is no evidence to show whether one or more pituitary hormones are involved.

In the intact animal *X*-zone degeneration may result from (*a*) reduced pituitary secretion or (*b*) direct action of excitant substances on the *X*-zone. Further, *X*-zone degeneration (i) at puberty in the intact male, (ii) during old age in castrated males and virgin females, (iii) during early pregnancy, may not be due to the same agency.

The available evidence may be summarized as follows.

Degeneration at puberty in the male. Degeneration in the intact male at puberty or in the immature male after gonadotropic injections is accounted for by the release of androgens into the circulation (p. 123). Whether androgens evoke degeneration by inhibiting hypophyseal activity or by direct action is unknown.

Degeneration in virgin females. In the absence of evidence to the contrary it seems probable that degeneration of the zone in virgin females is due to reduced secretion from the anterior lobe of the pituitary. This view is supported by degeneration of the zone in old males where gonadic influence has been excluded by early castration.

Degeneration during early pregnancy. There is no satisfactory explanation of *X*-zone degeneration during early pregnancy. Gonadotropic extracts injected into intact immature females cause degeneration of the *X*-zone. Degeneration does not occur after similar treatment of spayed animals [Takewaki, 1935]. It therefore seems probable that degeneration during early pregnancy results from the action of either (*a*) ovarian hormones alone or (*b*) ovarian hormones together with gonadotropin.

(*a*) Oestrone and progesterone have no effect in doses of physiological significance (pp. 125, 126). Starkey & Schmidt [1938] suggested that degeneration may be due to an ovarian androgen. This receives support from the observation that ovaries implanted into castrated males manifest androgenic activity. They maintain male accessory glands [Hill, 1937, 1941; Hill & Strong, 1938, 1940] and evoke degeneration of the

X-zone [Takewaki, 1939]. A difficulty this view must contend with is that ovaries implanted into females do not cause degeneration of the *X*-zone [Takewaki].

Alternative (*b*) has not been fully explored. The experiments described on p. 127 show that doses of oestrogens which alone do not evoke degeneration may do so when purified gonadotropin is administered at the same time. Apart from the fact that the doses administered are probably too high to be of physiological significance the findings are ambiguous for another reason. *X*-zone degeneration takes place in one of two ways. The cells may become highly vacuolated and eventually collapse, or fibrous degeneration without vacuolization may occur. With the former it is possible to discriminate clearly between degeneration of the *X*-zone by cytolysis of its constituent cells and degeneration due to pressure from an enlarging permanent cortex. With the latter it is not. In nos. 1–7 of Table 4 degeneration was of the second type.

It is further relevant to note that degeneration of the *X*-zone has been recorded in two classes of experiment in which there has been no attempt to relate the experimental findings to specific physiological events in the intact animal. (1) Gersh & Grollman [1939] observed that treatment with cortical extract caused *X*-zone degeneration and inferred that when the permanent cortex attains its full activity the *X*-zone is superfluous and degenerates. Howard [1940] criticized these experiments on the grounds that the extracts used may have contained androgens. She was unable to repeat their results using desoxycorticosterone. (2) Implantation of fresh pituitary and injections of vasopressin evoke degeneration of the *X*-zone in castrates. It is unlikely that the amount of vasopressin necessary to effect this response has any physiological significance.

A question of more general interest in regard to inhibition of hypophyseal activity is raised by the results of oestrogen treatment. Moore & Price [1932] showed that the small dose of 6 rat units of oestrogen per day for 20 days was sufficient to cause severe tubular damage in the testes of 30-day-old rats. Doses of 10–15 r.u. per day for the same period caused similar damage in mature males. Parallel injection of mixtures of oestrogens and gonadotropin showed that damage caused by the former was due to inhibition of gonadotropic activity of the intact pituitary. The present experiments show that much larger quantities do not cause destruction of the *X*-zone. So it seems that oestrogens do not readily inhibit release of the pituitary principle necessary for maintenance of the *X*-zone. Other evidence indicates that oestrogens do not inhibit secretion of the pituitary principle responsible for the maintenance of the permanent cortex. It seems more likely that the release of the latter is facilitated. Except when treatment is prolonged oestrogens cause *enlargement* of the permanent cortex [summary of literature in Deanesly, 1939]. This effect may be attributed to (*a*) direct effect of oestrogens on the adrenal cortex or (*b*) an indirect effect through the pituitary. Since cortical hypertrophy does not occur in hypophysectomized animals the latter is the more likely.

SUMMARY

1. Oestrone and progesterone separately or together do not cause degeneration of the *X*-zone of the mouse adrenal cortex.

2. Injection of pregnancy urine gonadotropin after oestrone causes *X*-zone degeneration in castrated males.

3. Implantation of whole pituitaries and also injection of large doses of vasopressin voked degeneration in a small group of castrates.

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THE KETOGENIC ACTIVITY OF EXTRACTS OF THE ANTERIOR PITUITARY

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It is now well recognized that fasting rats are more suitable than fat-fed rats for the determination of the ketogenic activity of extracts of endocrine organs [Shipley & Long, 1938; Black, Collip & Thomson, 1934; Gray, 1938]. Shipley & Long found that measurements of the increases in ketonaemia were more consistent than those of increases in ketonuria in such investigations.

The experiments described here deal with modifications and improvements in the mode of assay previously described [Gray, 1938] and with the application of such improved methods to the determination of ketogenic potency of material prepared by ammonium sulphate fractionation of extracts of the anterior lobes of ox pituitaries.

In view of the findings of Shipley & Long [1938] that the ketone bodies are threshold substances, a number of assays were checked by using the method described by these workers. In addition, a number of experiments were performed in which the effect of ketogenic extracts on ketonaemia and ketonuria were simultaneously investigated. An attempt has been made to correlate the findings in order to interpret rises in ketonuria in terms of rises in ketonaemia.

Finally, the ketogenic activities of extracts from ox and sheep anterior lobes of the hypophysis were compared.

METHODS

Biological

Investigation of ketonuria

Male rats weighing 100–150 g. were employed. They were fasted for 36 hr. before injection of the extracts to be tested. After injection they were placed in individual metabolism cages and the urine collected during the following 24 hr. 10 ml. of water were administered by stomach tube every 4 hr. for the first 12 hr. during the period of urine collection. Finally, the cages and the funnels were washed in a standard manner with 10 ml. of water. In this way each rat yielded 17–30 ml. of urine with washings, a quantity suitable for macro-analysis.

Investigation of ketonaemia

The method of Shipley & Long has been carefully followed.

Simultaneous investigation of ketonuria and ketonaemia

Numerous attempts were made to determine the ketones excreted in 4 or 8 hr. and thus to correlate these with the blood ketone concentration measured during that period. Owing to the difficulty of obtaining urinary specimens suitable for analysis, this was soon found impracticable. In the experiments recorded below the rats after fasting for 36 hr. were bled from the tail vein, the extract was injected

subcutaneously, and urine collected during the following 24 hr. A second sample of blood was taken from the tail vein 4 hr. after the injection. The ketone-body contents of the two blood samples and of the 24 hr. output of urine were determined. Urinary output was maintained as before by the administration of three 10 ml. quantities of water by stomach tube.

Chemical

Preparation of extracts

These were very kindly provided by Dr F. G. Young of the National Institute for Medical Research, London. Their preparation and hormonal potencies have been described elsewhere [Young, 1938 *a*, *b*, *c*, 1939; Marks & Young, 1940]. All the extracts were kept in the frozen state until shortly before use.

Chemical estimations

Urinary ketones were determined by the Denigès-Van Slyke method [Peters & Van Slyke, 1932]. With very little experience, it was possible to judge when the mercuric sulphate-acetone precipitate corresponded to less than 1 mg. of acetone per 24 hr. In such cases the precipitate was not weighed but recorded as corresponding to 0 mg./24 hr. Blood ketones were in a few instances determined by the Engfeld [1925] method, but this was soon abandoned in favour of the modification of the Scott-Wilson method described by Shipley & Long. The distillation apparatus was modified so that it could be constructed from standard Quickfit and Quartz apparatus with standard glass-joints. All the ketonaemia results recorded here were carried out by this method.

RESULTS

In all, 132 simultaneous determinations of ketonaemia and ketonuria in injected and uninjected rats have been made and the results are summarized in Table 1. For

Table 1. *Ketonaemia and ketonuria*

	Rats injected with doses of extract effective in inducing rises in blood ketones > 5 mg./100 ml.			Control rats and rats injected with doses of extract ineffective in inducing rises in blood ketones > 5 mg./100 ml.		
Blood ketones mg./100 ml.	No. of rats	Average ketonuria mg./24 hr. ± S.D.	Range mg./24 hr.	No. of rats	Average ketonuria mg./24 hr. ± S.D.	Range mg./24 hr.
0 - 4·9	0	—	—	13	1 ± 1	0- 3
5·0- 9·9	1	0	0	16	0·5 ± 0·7	0- 2
10·0-14·9	15	6 ± 6	0-17	14	1 ± 1·4	0- 5
15·0-19·9	11	15 ± 16	0-40	3	9 ± 2	6-12
20·0-24·9	27	11 ± 12	0-51	1	1 ± 0	1
25·0-29·9	8	22 ± 30	0-85	0	—	—
30 -34·9	11	18 ± 22	0-76	0	—	—
Over 35	12	15 ± 15	0-46	0	—	—

reasons which will become obvious later, the results have been divided into two groups. Those from rats which responded to injections of extracts of the anterior pituitary by increases in the blood ketones of 5 mg. or more per 100 ml. are separated from the results from controls and rats which failed to respond to the injections in this way.

Detailed analysis of the results showed that the increases in ketonaemia occurred more regularly than the increases in ketonuria in rats injected with adequate doses

of potent extract, but even with the measurements of ketonaemia there was great variability in response. The existence of a renal threshold for ketone bodies was clearly demonstrated but it was also obvious from the results that this threshold must vary greatly from rat to rat. Thus, in all, twenty-three rats were encountered with blood ketones which rose above 30 mg./100 ml., and of these, six produced a ketonuria of less than 4 mg./24 hr. These six must have had a renal threshold of more than 30 mg./100 ml. On the other hand, in a series of seventy-three rats with blood ketones below 20 mg./100 ml., twenty-three produced ketonuria of 4 mg. or more per 24 hr. (The highest ketonuria observed in a rat with a ketonaemia below 20 mg./100 ml. was 40 mg./24 hr.) These twenty-three rats must have had renal thresholds below 20 mg./100 ml. With blood ketones below 10 mg./100 ml. the ketonuria was never above 4 mg. in 24 hr. The results of Shipley & Long suggest that the renal threshold for ketone bodies was fairly constantly between 25 and 30 mg./100 ml. This has by no means been the case in the series recorded here.

It is therefore obvious that with a fixed dose of a potent extract at least three variable factors must be concerned in influencing the magnitude of the ketonuria induced. The factors comprise the initial level, the rise of the blood ketones and the renal threshold.

Oastler & Anderson [1939] found that the renal threshold for ketone bodies was raised after hypophysectomy, and it was therefore thought possible that injections of pituitary extracts might lower the renal threshold. No conclusive evidence has been obtained to prove or disprove this possibility, but the results in Table 1 with the limited number of experiments available suggest that the renal threshold may well be lowered by the injection of pituitary extracts. Thus in control rats and in rats in which the blood ketones were raised by less than 5 mg./100 ml., no appreciable ketonuria occurred except with a blood-ketone level of more than 15 mg./100 ml. On the other hand, in rats which responded to pituitary extracts by increases in ketone bodies of more than 5 mg./100 ml., significant ketonuria was observed in rats with blood ketones lying between 10 and 14·9 mg./100 ml. There was, however, considerable variability.

If pituitary extracts lower the renal threshold for ketone bodies it would be expected that the increases in ketonuria would be rendered more uniform. This on the whole is reasonably true for large doses of extract which produced significant increases in ketonuria in all rats within a group of five. In such groups the rises in blood ketones were much smaller than those occurring with smaller doses of extract which caused only a few rats within a group of five to produce a significant increase in ketonuria. In the latter groups it might well be that the ketogenic effect is present without the renal threshold-lowering effect. Thus a large dose of extract would decrease the renal threshold so that the blood ketones were unable to reach such a high level as when smaller doses were used, and which did not result in this lowering of renal threshold.

It is agreed that the ketonaemic responses although somewhat variable are considerably less variable than the ketonuric responses, especially when the dose of extract injected is minimal. However, a large number of assays of ketogenic activity of anterior pituitary extracts had been made before the ketonaemic method was used. It was intended to repeat all of these assays using the ketonaemic method,

but present conditions have prevented this. It has therefore been necessary to determine some way of interpreting these ketonuric responses, particularly those in which only a few rats within the group produced excess ketone bodies in the urine. Table 2 summarizes the results obtained and shows that if any one rat within a

Table 2. *Relation between increases in blood-ketone bodies and ketonuria*

Increase in ketonaemia mg./100 ml.		No. of rats in group	No. of rats in group producing more than 4 mg./24 hr.
Average ± S.D.	Range		
27·9 ± 19·0	5·7–74·7	10	6
20·0 ± 5·8	15·2–28·6	5	3
18·8 ± 9·4	5·9–40·3	10	8
18·5 ± 3·3	15·2–24·6	5	5
16·7 ± 12·5	1·5–37·0	9	2
15·6 ± 7·9	3·8–16·6	5	3
14·6 ± 7·4	3·7–25·6	5	2
14·4 ± 7·5	11·2–17·9	5	2
13·2 ± 5·2	10·1–24·0	6	2
13·2 ± 5·5	6·6–21·2	10	10
11·4 ± 4·0	6·2–18·6	5	5
10·7 ± 8·5	0·2–23·0	5	3
9·6 ± 11·7	−0·7–23·6	5	3
9·4 ± 2·0	5·9–12·0	5	5
7·7 ± 7·2	−0·2–20·0	5	0
6·5 ± 2·7	4·1–11·7	5	3
5·7 ± 1·6	3·0– 7·6	5	1
−1·8 ± 1·6	−5·3– 2·3	5	0
0·8 ± 3·3	−5·3– 2·3	5	0
−0·8 ± 2·6	−5·6– 2·7	5	0

series of five produces a ketonuria of more than 4 mg. in 24 hr. following the injection of an extract, that extract will very probably, if not certainly, have raised the blood ketones by a significant amount. If no rat within the group of five excretes more than 4 mg. in 24 hr., then the extract may possess weak ketogenic activity as based on the ketonaemic method. Table 2 also shows the ketonaemic responses obtained in groups of rats which all produced significant increases in ketonuria. It will be seen that the mean increases in ketonaemia are considerably lower than those occurring in groups of rats which do not all produce such amounts of ketone bodies.

There is another point of discrepancy between these results and those of Shipley & Long. They found that rats with an initial fasting level of blood ketones of about 3 mg./100 ml. or lower were relatively unresponsive, whereas in this series such rats responded quite well to effective doses of potent extract.

Ketonuria

Considerable variations in the magnitude of the ketonuria elicited by extracts of the anterior pituitary were observed. This is in accord with the results of Shipley & Long [1938]. Previous to the publication of their results demonstrating a renal threshold for ketone bodies in the rat, attempts were made in other directions to find the cause of these uneven responses.

Rats were used in considerable numbers, and it became necessary to supplement the supply from the King's College Hospital colony with animals from the Glaxo Laboratories and from the Medical Research Council farm. Those supplied from the

latter source were found to be relatively unresponsive in that the ketonuria produced by extracts of the anterior pituitary were always very much smaller than that produced by the same extracts in animals from the other sources. Attempts were made to correlate these findings with the previous diet, but although the M.R.C. animals were put on supplements of protein, fat and vitamin B_1 for 2 or 3 weeks before the actual assay of ketogenic extracts, the animals remained relatively unresponsive to the action of extracts known to be active in animals from the other two sources. At one time it appeared that there was a seasonal variation in the ability of the rat to produce ketone bodies in the urine in response to such extracts, but experience extending over 5 years has not succeeded in proving this possibility. In view of these results, animals from the King's College Hospital colony and the Glaxo Laboratories only have been included in the series of experiments described here.

The ketogenic activity of prolactin and non-prolactin fractions of the anterior pituitary gland

Both the *p*H 5·5-soluble fraction (non-prolactin C of Young [1938*b*]) and the *p*H 5·5-insoluble fraction (prolactin C) were active in inducing marked ketonuria in the fasting rat. The former fraction was active in doses down to 0·1 ml. (≡25 mg. of fresh gland ≡ approximately 0·2 mg. of solid material). At this dosage the average ketonuric response was 19 mg. of ketone bodies per 24 hr.

The *p*H 5·5-insoluble fraction was similarly active with a dose of 1 ml. (≡250 mg. of fresh tissue ≡ approximately 2 mg. of solid material). Insufficient *p*H 5·5-insoluble material was available for complete testing purposes, but the general impression gained was that it was active but less so than the *p*H 5·5-soluble fraction.

*The ketogenic activity of euglobulin, pseudoglobulin and albumin fractions of the p*H 5·5*-soluble material*

In all euglobulin, pseudoglobulin and albumin fractions of six different preparations of the *p*H 5·5-soluble material were assayed, and the results are summarized in Fig. 1. It will readily be seen that all the fractions are active in inducing ketonuria in the fasting rat and that there is no preferential precipitation of the ketogenic factor in any of the fractions, although the variability of the responses would tend to mask any slight differences in the potencies of the various fractions.

*The ketogenic activity of the previous fractions heated at p*H 10 *for* 15 *min.*

Fig. 1 also shows the ketonuria induced in fasting rats by the euglobulin, pseudoglobulin and albumin fractions of the *p*H 5·5-soluble material after heating at *p*H 10 for 15 min. In about two-thirds of the experiments the ketonuria was completely abolished; in the remainder, there was a residual activity which was very much less than that of the unheated fractions.

A few experiments were carried out with extracts which had been allowed to stand for 24 hr. at room temperature. These stale extracts usually showed slight diminution in ketogenic activity.

The ketogenic activity of fractions prepared from sheep anterior pituitaries

Fig. 1 further shows the ketonuria induced in fasting rats by the globulin and albumin fractions of saline extracts of sheep pituitaries. It will be seen that these fractions are as active as those prepared from saline extracts of ox pituitaries.

Ketogenic activity of fractions prepared from acetone-dried glands

Extracts prepared from B.D.H. acetone-dried glands with fractionation by the methods described by Young [1938*b*] were assayed and found to be weakly active

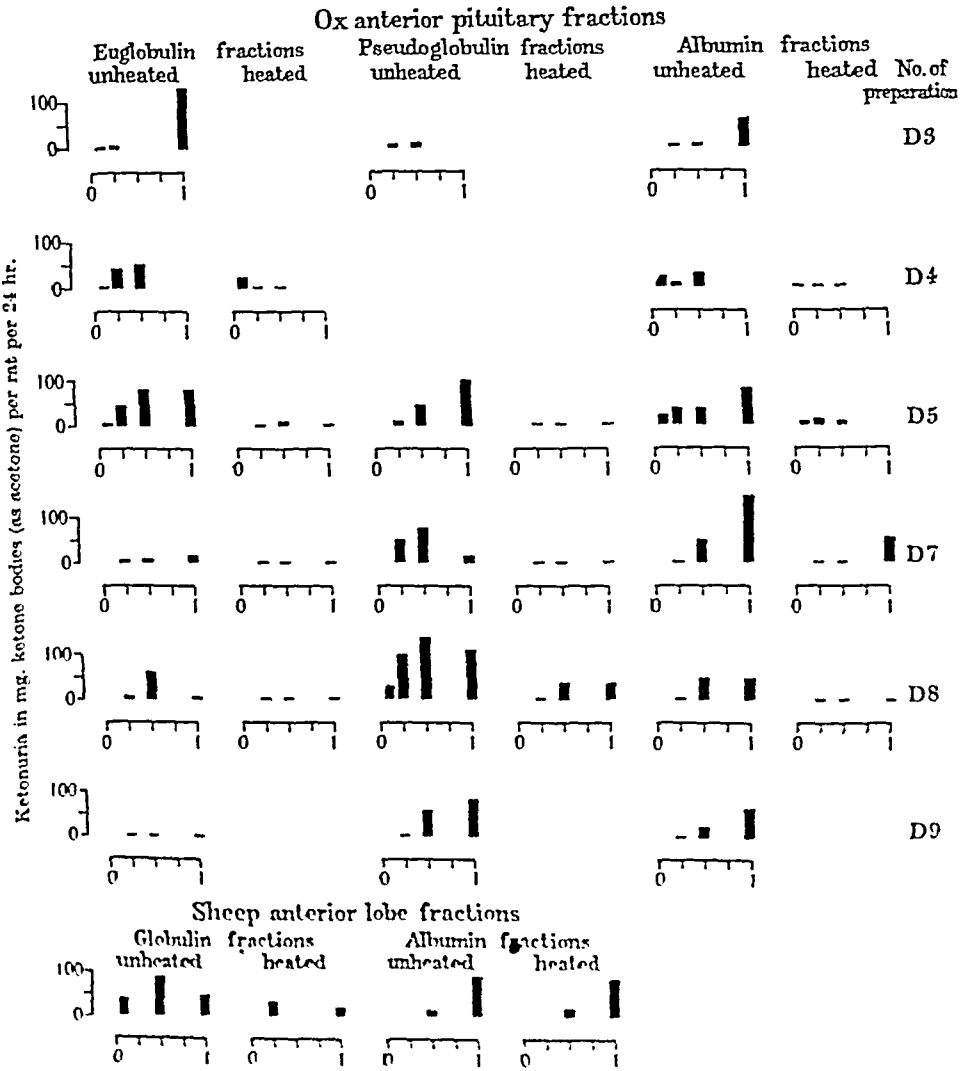


Fig. 1. Ketonuria results (each ketonuria result is the mean obtained from five or more rats)

in increasing the ketonuria of fasting rats. The fractions otherwise were much less active than were those prepared from fresh glands.

Ketogenic activity as determined by measurement of ketonaemia

The results of the assays for ketogenic activity of pseudoglobulin, euglobulin and albumin fractions of saline extracts using the method of Shipley & Long are shown in Fig. 2. It has not been possible to assay the albumin fraction as fully as the others. As far as they go the results would indicate that the albumin fraction is less active than the globulin fraction, but it must be emphasized that considerably fewer animals were used in the assay of the albumin fraction than in that of the other fractions. The result of the assay using ketonuria as a criterion of ketogenic

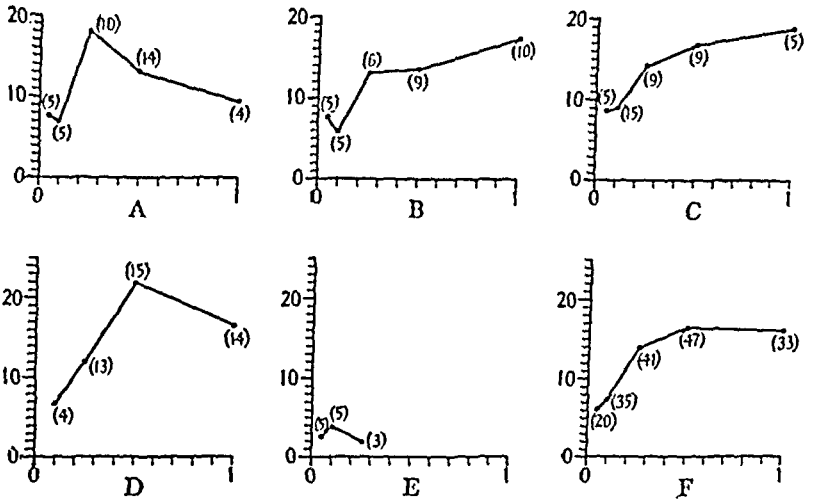


FIG. 2. Ketonaemia results. Ordinates: increase in blood ketones (as acetone) in mg./100 ml. Abscissae: dose of extract in ml. 1 ml. of saline extract≡0·25 g. of fresh anterior lobe; 1 ml. of fractionated extract≡0·5 g. Figures in brackets give the number of results of which the mean is recorded. Curve *A*, crude saline extract of ox pituitary. Curve *B*, crude saline extract of sheep pituitary. Curve *C*, euglobulin fraction of saline extract of ox pituitary. Curve *D*, pseudoglobulin fraction of saline extract of ox pituitary. Curve *E*, albumin fraction of saline extract of ox pituitary. Curve *F*, composite curve of all ketonaemia results.

potency, shows that while the albumin fraction occasionally has less action than the euglobulin-pseudoglobulin fractions, this is not a constant feature, and some albumin fractions appear even more ketogenic than the others.

On the whole, the evidence indicates that ketogenic activity is not precipitated preferentially with any fraction.

Saline extracts prepared from sheep anterior pituitaries were also assayed and were found to be as active as saline extracts from ox anterior pituitaries.

DISCUSSION

The lack of uniformity in the renal threshold for ketone bodies is a disturbing feature of this series of experiments, in view of the comparative regularity of this factor in the experiments described by Shipley & Long. The renal threshold-lowering effect of *large* doses of anterior-pituitary extract would certainly explain this variation in most cases but not in all, since in a very occasional uninjected rat a considerable ketonuria is observed with a relatively low level of blood ketones. The results summarized in Table 2 merely indicate the possibility of this renal threshold-lowering

effect, but the point cannot be regarded as proved conclusively owing to the relatively small number of animals with blood ketones within the necessary range. Present conditions have prevented further work on this matter. The striking differences in the ketonuria induced by pituitary extract in rats from different sources point to the possibility of unknown hereditary factors playing a part in producing such diverse results, although the rats used in both Shipley & Long's and this work were of Wistar strain.

A renal threshold-lowering effect of *large* doses of anterior-pituitary extract would tend to produce a smaller increase in blood ketones and would tend to make the increases in the ketonuria more uniform, both of which effects have been observed fairly regularly.

A further point which does not seem in the past to have been sufficiently emphasized is that the rat is unusual in not developing a ketonuria after a 48 hr. fast. In man, the dog and the cat such a period of fasting usually results in a considerable ketonuria. Accordingly, results of experiments on ketosis in the rat should be interpreted with great care, and should not be used to elucidate problems of ketosis in other species.

There is little doubt that crude saline extracts and the crude thyrotropic fractions (non-prolactin C) of the anterior pituitary are more active in inducing ketosis than any of the fractions derived from them by the methods described by Young [1938 *a*, *b*, *c*]. Fractionation into euglobulin, pseudoglobulin and albumin fractions appears to result in the ketogenic activity being distributed fairly evenly among the three fractions. If anything, the euglobulin fraction was slightly more active than the other two fractions, but this was by no means a constant observation.

Table 3. *Biological activity of anterior-pituitary extracts*

	Biological activity							
		Gonadotropic*						
Extract	Thyrotropic	*a*	*b*	Prolactin	Glycotropic	Diabetogenic	Growth promoting	Ketogenic
(*a*) *From fresh ox anterior lobes*								
Crude saline	+ + +	+ + +	Trace	+ + +	+ + +	+ + +	+ + +	+ + +
Non-prolactin *p*H 5·5-soluble	+ + +	+ + +	Trace	Trace	+ +	+ +	+ +	+ + +
Prolactin *p*H 5·5-insoluble	Trace	Trace	0	+ + +	+ +	0	+	+ +
Euglobulin fraction of non-prolactin fraction	+	Trace	Trace	0	+	0	Trace	+ +
Pseudoglobulin fraction of non-prolactin fraction	+ +	+ +	Trace	0	+	+	+	+ +
Albumin fraction	+	+ +	Trace	0	+	0	0	+ + (? +)
(*b*) *From B.D.H. acetone-dried gland*								
Pseudoglobulin + albumin fraction	+ +	0	0	+ +	+	0	+	+
Prolactin fraction	0	0	0	+ +	+	0	+	+
All heated fractions	0	0	0	Trace	+	0	0	±
(*c*) *Sheep extracts*								
Crude saline and euglobulin and albumin fractions	All fractions possess much the same activity as corresponding fractions from ox anterior lobe							

a = capacity to cause ovulation in oestrous rabbits
b = capacity to increase ovarian weight in immature rats

Table 3 summarizes the activities of the various extracts which have been tested for ketogenic activity. It will be seen that the ketogenic activity does not appear to run parallel with any of the other biological activities, although, of course, this does not preclude the possibility that ketogenic activity is merely one component of a complex which can produce the other biological effects [see Young, 1938 *a*, *b*, *c*]. If anything, the distribution of ketogenic activity runs parallel with the distribution of glycotropic activity, but the partial heat-lability of the former as opposed to the heat-stability of the latter indicates that the two factors may be quite separate. However, experiments in connexion with the assay of such crude extracts for biological activities must be interpreted with very great caution. Thus it is possible that the glycotropic factor or ketogenic factors are identical. If the ketogenic activity is secondary to the glycotropic effect and the methods of assay for ketogenic factors are less sensitive (as may well be the case) than methods for assay of glycotropic activity, then one might well observe an apparent dissociation of the two activities.

SUMMARY

1. The relationship between ketonuria and ketonaemia in the fasting rat injected with saline or injected with extracts of anterior lobes of the pituitary has been investigated.

2. The findings of Shipley & Long [1938] that there is a renal threshold for ketone bodies in this species has been confirmed, although the actual *level* was not found to be so constant as reported by them.

3. It is considered possible that injections of extracts of the anterior lobe of the pituitary into fasting rats may lower the renal threshold for ketone bodies in the rat.

4. The ketogenic activity is distributed fairly evenly among the albumin, globulin and pseudoglobulin fractions of *p*H 5·5-soluble extracts of the anterior pituitary.

5. The ketogenic activity of *p*H 5·5-soluble fractions of saline extracts of the pituitary is nearly as great as the crude saline extracts from which they are derived.

6. The ketogenic activity of such fractions runs parallel with the glycotropic activity alone, but the heat-stability of the latter indicates that the two factors may be separable.

The author wishes to express his gratitude to Dr F. G. Young for all the extracts of anterior pituitary tissue described and for the data of Table 3, apart from the ketogenic activity. Thanks are also due to Miss M. Sandiford and Mr A. H. Dawson for much assistance, and to the Medical Research Council for an expenses grant.

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OOGENESIS AND ITS RELATION TO THE OESTROUS CYCLE IN THE ADULT MOUSE

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It is now evident, at least in those species which have been closely studied, that new crops of oogonia are produced cyclically by the ovaries of adult vertebrates, and that the old theory of the laying down of an irreplaceable stock of young eggs during embryonic life must be abandoned. In the lower vertebrates these facts have been accepted for some time, although there has been some mystification concerning the source and mode of origin of the new eggs. Wheeler [1924] suggested that in the dab, *Pleuronectes limanda* L., they were produced after each breeding season from the cells of the collapsed follicles, but it seems, as in the minnow, *Phoxinus laevis* L. [Bullough, 1942*a*], that it is the common condition for new oogonia to arise from the mitotic divisions of the cells of the germinal epithelium investing the ovary. In the warm-blooded vertebrates the fact that new eggs are produced cyclically throughout adult life is still not generally accepted in spite of the fact that it has been demonstrated in one bird [Bullough & Gibbs, 1941; Bullough, 1942*b*] and in many mammals [see review of Swezy, 1933].

The production of new oogonia in the adult vertebrate ovary is commonly stated to be a rhythmic phenomenon connected with the reproductive cycle, and it appears probable that some hormonal mechanism is the controlling factor. In animals like the minnow and the starling, *Sturnus vulgaris* L., which usually breed only once in the year, the production of new oogonia is also a yearly occurrence, but in polyoestrous mammals, like the mouse, it takes place most actively at each oestrous period [Allen, 1923]. As a first step towards the elucidation of the mechanism controlling these rhythms, the present paper is a record of a detailed study of the mitotic activity of the germinal epithelium of the mouse during the various phases of the oestrous cycle.

MATERIAL AND METHODS

The forty mice used in these experiments were of several strains, but all were characterized by steady and regular oestrous cycles. The type of mouse appeared to have little or no effect on the results obtained, and neither did the fact that, previous to the experiments, some had been mated and had reared families whereas others had not. The animals were kept in uniformly warm conditions, were exposed to 12 hr. of light each day both before and during the experiments, and were given a varied diet including grain, dog biscuit, milk, cod-liver oil and lettuce. The experiments were performed in the spring and summer of 1941.

The vaginal smear technique was used to distinguish the phases of the oestrous cycle, smears being taken from each animal twice daily for at least 10 days before

killing. The smears were stained in Ehrlich's haematoxylin and Pasini's stain, which was found to give much more precise results than normal counter-stains such as eosin. The following stages of the oestrous cycle were recognized:

(1) Pro-oestrus; only blue-staining nucleated epithelial cells present.

(2) Pre-ovulation oestrus, early; some cells cornified with cytoplasm staining red. Pre-ovulation oestrus, full; all cells cornified and staining bright red throughout.

(3) Post-ovulation oestrus; cornified cells in clumps and pale pink in colour.

(4) Metoestrus; leucocytes staining very dark blue among the pale pink cornified cells.

(5) Dioestrus, first day; first signs of blue-staining nucleated epithelial cells, leucocytes still more numerous, and traces of pale pink cornified cells usually still persisting. Dioestrus, second day; only blue-staining nucleated epithelial cells and leucocytes. Dioestrus, third day; as second day.

With the staining method used, still further subdivision of these stages was possible, but this proved unnecessary for the present purposes.

To facilitate the study of the waves of mitoses in the cells of the germinal epithelium, the colchicine technique was used to arrest the cell divisions in the metaphase. The method adopted was that described by Allen [1937], each animal being given a subcutaneous injection of 0·1 mg. of colchicine in 0·25 ml. of water 9½ hr. before killing. The mice were anaesthetized with chloroform, the body cavity, thorax, and throat were opened, and the whole bodies were fixed for 2 days in Bouin's solution. The ovaries were then removed, and sections were cut to a thickness of 7μ. The nuclei were stained with Ehrlich's haematoxylin, the cytoplasm with a saturated aqueous solution of eosin, and the connective tissue with a saturated solution of saffron in absolute alcohol.

In examining the slides for mitoses of the germinal epithelial cells, an attempt was made to count every one of these divisions in each ovary. As parts of each mitosis were usually found on two adjacent sections, the counts were made only on every other section, and it is believed that very few mitoses were missed. All the cell divisions were classified at the time of counting according to their proximity to the following structures.

(1) Groups of very small primary follicles or masses of stroma and interstitial cells.

(2) Rapidly growing follicles containing follicular fluid.

(3) Developing corpora lutea not yet luteinized.

(4) Fully developed corpora lutea not more than one oestrous cycle old.

(5) Corpora lutea more than one oestrous cycle old.

The separation of groups 4 and 5 was mainly on the basis of depth of staining with eosin. The cytoplasm of the older corpora lutea stained a deeper red than that of the younger ones. The definition of what was considered to constitute proximity to the various structures is illustrated in Fig. 1. In the case of a follicle, the adjacent germinal epithelium was that found between the shortest lines drawn at right angles to the surface of the ovary and forming tangents to the sides of the follicle. The same rule was applied to the corpora lutea, and all the remaining germinal epithelial cells, having no relation to anything except the very small follicles or the ovarian stroma and interstitial cells, were included in the first group.

The counts of mitoses in the ovaries of each group of mice were averaged, and the standard deviation from the mean was calculated according to the formula recommended by Simpson & Roe [1939] for use with small samples.

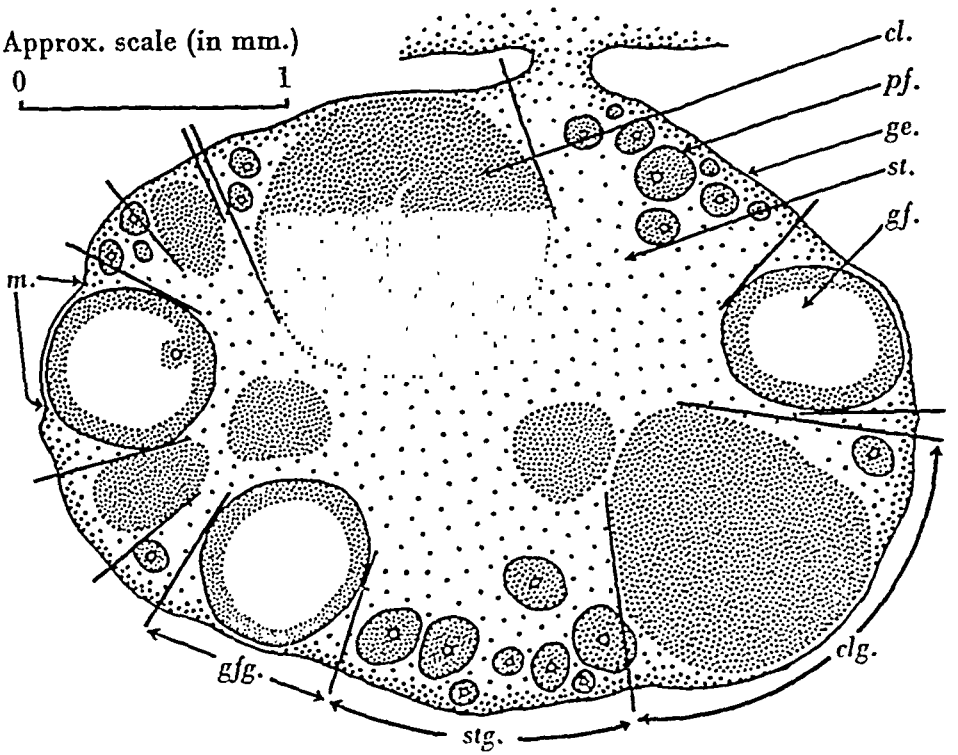


FIG. 1. Section of a mouse ovary showing the method of determining the regions of germinal epithelium adjacent to the various ovarian structures. *Cl.* corpus luteum; *clg.* germinal epithelium adjacent to corpus luteum; *ge.* germinal epithelium; *gf.* growing follicle, *gfg.* germinal epithelium adjacent to growing follicle; *m.* region of greatest mitotic activity in germinal epithelium, *pf.* primary follicle; *st.* ovarian stroma; *stg.* germinal epithelium adjacent to small primary follicles and ovarian stroma.

OBSERVATIONS

The mouse ovary, completely enveloped in a connective tissue capsule, is covered by a continuous germinal epithelium, usually only one cell thick, which is based on a thin connective tissue layer, the tunica albuginea. It was found that the cells of the germinal epithelium vary considerably in shape according to the degree of tension. Those stretched across large follicles or corpora lutea were usually squamous, whereas those covering the remainder of the ovary surface were usually cubical or even columnar in form. Cells of all forms were seen to undergo mitosis, but division was especially common among those which were cubical or columnar. Each cell, after entering the prophase, swelled to many times its original volume, and at the same time the cytoplasm lost its affinity for stain. Both these processes were complete when the metaphase was reached. The swollen cell then usually protruded from the ovary surface, and the clearness of the cytoplasm, together with the darkly staining

mass of chromosomes, made it very conspicuous. Allen [1923] has described how the two daughter cells, according to the plane at which the division takes place, form either oogonia, follicle cells, or new cells of the germinal epithelium.

In each of the phases of the oestrous cycle, one ovary from each of four mice was examined in detail. The results obtained are presented in Table 1, in which the relations of the mitoses to the various ovarian structures are also indicated, and in the graph (Fig. 2). In the pro-oestrous period the total number of mitoses in the four ovaries varied between 66 and 98, and it was clearly evident that they were most common in the restricted areas of the germinal epithelium close to the rapidly growing follicles. They also occurred in small numbers, however, over the whole of the rest of the germinal epithelium, either close to the corpora lutea or with no relation to any structure other than very small follicles or ovarian stroma.

Table 1. *Average numbers of mitoses of the germinal epithelial cells classified according to their proximity to various structures in the ovaries of mice killed in different phases of the oestrous cycle*

	Mitoses						
Phase of oestrous cycle	Unrelated ar. ± σ*	By large follicles ar. ± σ	By developing corpora lutea ar. ± σ	By young corpora lutea ar. ± σ	By old corpora lutea ar. ± σ	Total ar. ± σ	No. of ovaries n
Pro-oestrus	27·2 ± 6·4	41·2 ± 4·2	—	5·5 ± 3·1	7·5 ± 4·2	81·5 ± 15·8	4
Oestrus:							
Pre-ovulation	28·7 ± 4·6	75·2 ± 12·5	—	4·5 ± 4·4	4·0 ± 3·6	112·5 ± 24·4	4
Post-ovulation *a*	117·5 ± 14·2	8·7 ± 11·8	497·2 ± 84·2	—	11·6 ± 3·9	635·0 ± 96·2	4
Post-ovulation *b*	192·7 ± 38·3	7·2 ± 8·8	997·0 ± 203·7	—	13·2 ± 2·2	1210·0 ± 228·9	4
Metoestrus	12·7 ± 6·1	14·7 ± 12·2	—	66·2 ± 18·1	6·0 ± 6·1	100·0 ± 37·2	4
Dioestrus:							
First day	9·7 ± 7·7	4·7 ± 5·7	—	4·7 ± 4·9	0·7 ± 1·5	20·0 ± 19·4	4
Second day	3·7 ± 2·5	5·5 ± 2·4	—	10·0 ± 7·7	4·0 ± 2·9	23·2 ± 12·3	4
Third day	5·7 ± 3·1	14·8 ± 5·3	—	6·5 ± 3·1	3·8 ± 1·9	30·8 ± 10·5	4

* ar. = arithmetic mean of the number of mitoses, σ = standard deviation = $\sqrt{\frac{fd^2}{n-1}}$, where n = number of ovaries examined.

The largest number of mitoses of the germinal epithelial cells occurred in the oestrous phase, but in order to emphasize the extreme shortness of the period of maximum activity, it was necessary to subdivide oestrus into pre- and post-ovulation periods. The pre-ovulation oestrous period had an average duration of about 36 hr., and showed far fewer mitoses than the post-ovulation oestrous period. However, more mitoses were evident than in pro-oestrus, and in the four ovaries examined the numbers varied between 93 and 149. The association of most of these with the almost fully grown follicles was most marked. They were unusual among the squamous epithelial cells covering the follicles, although they did occur in this region, but they were common in a narrow circular area (marked *m.* in Fig. 1) immediately surrounding the follicle. In this area the germinal epithelial cells were usually either cubical or columnar in form.

The number of mitoses rose very rapidly to a peak in the short post-ovulation oestrous period. This phase, still typified by a fully cornified vaginal smear, lasted for about 12 hr., and was further subdivided according to the appearance of the bufst follicle, now termed the developing corpus luteum, into two parts each of about 6 hr. duration. In the first of these (post-ovulation *a*) the follicle had only recently burst (Plate 1, fig. 3), the old follicle cells hung out from the surface of the ovary, and the follicular fluid had escaped into the periovarian space. The germinal epithelium, which had covered the follicle, was broken and retracted into a circular

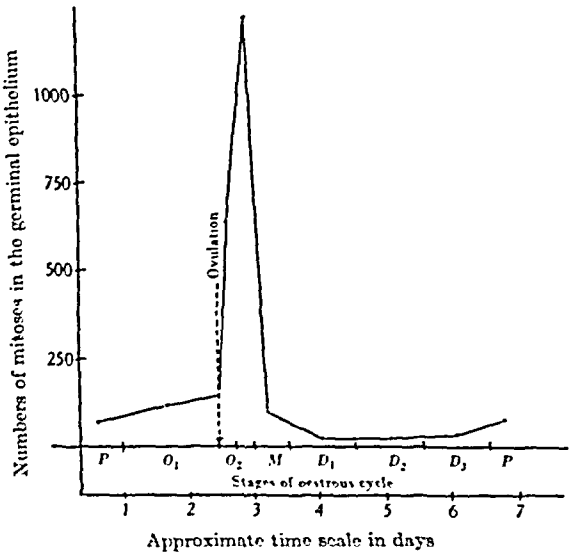


FIG. 2. Graph showing the average number of mitoses in the germinal epithelium of one ovary at each of the various stages of the oestrous cycle. *P*, pro-oestrus; O_1, pre-ovulation oestrus; O_2, post-ovulation oestrus which is subdivided into two periods, early and late; *M*, metoestrus; D_1, first day dioestrus; D_2, second day dioestrus; D_3, third day dioestrus.

area (marked *m*. in Plate 1, fig. 3), and it was in this narrow area that the mitoses were so common. The total numbers of mitoses in each of the four ovaries examined in the early post-ovulation phase varied between 499 and 706.

In the course of a few hours the old follicle cells were drawn back into the ovary, and the covering of germinal epithelial cells was reformed. For a short time follicular fluid continued to be produced, and a new reservoir appeared in the centre of the mass of cells forming the young corpus luteum (Plate 1, fig. 4). This appearance was accepted as the criterion of the second of the two post-ovulation oestrous phases (post-ovulation *b*). In this short period the greatest numbers of mitoses of the germinal epithelial cells were found. The extreme counts in the four ovaries examined were 901 and 1432, and by far the greatest proportion of the mitoses was situated in the close proximity of the developing corpora lutea. The flattened epithelial cells covering the young corpora lutea were commonly seen in mitosis, but again the greatest number of divisions was present in the narrow rings of cuboidal cells round the edges (marked *e*. in Plate 1, figs. 3, 4). Mitoses of the epithelial cells were [illegible]

found over the whole surface of the ovary, on the sides of the hilus, and in the region close to the hilus where the germinal epithelium merged into part of the peritoneum. The ovaries of several animals killed in the post-ovulation oestrous periods contained one or two large follicles which had failed to burst. As in the case of the follicles in the pre-ovulation oestrous period, these had mitoses associated with them, and again, they were almost all situated in the narrow circular zone surrounding the bulge caused by the follicle.

The connexion between the mitoses of the germinal epithelial cells and the growing follicles was further illustrated by mitosis counts on the ovaries of mice which, in the pre-ovulation oestrous period, had produced either unusually small or unusually large numbers of follicles. These mice (nos. 13, 251 and 346) were not used when compiling Table 1, but they are included in Table 2 together with the more normal

Table 2. *Numbers of mitoses in the germinal epithelium correlated with the numbers of large follicles in the ovaries of mice killed in the pre-ovulation oestrous period. Each set of counts represents one ovary*

	Mitoses					
No. of mouse	Unrelated	By large follicles	By young corpora lutea	By old corpora lutea	Total	No. of follicles
13	8	22	1	1	32	2
251	14	40	3	5	62	3
14	32	68	1	0	101	4
131	29	70	3	5	107	5
12	18	69	3	3	93	6
83	36	94	11	8	149	7
346	54	137	4	6	201	11

Table 3. *Numbers of mitoses in the germinal epithelium correlated with the numbers of large follicles and developing corpora lutea in the ovaries of mice killed in the second post-ovulation oestrous period. Each set of counts represents one ovary*

	Mitoses					No. of	
No. of mouse	Unrelated	By large follicles	By developing corpora lutea	By old corpora lutea	Total	Large follicles	Developing corpora lutea
85	136	18	735	12	901	2	4
252	215	11	946	16	1188	1	5
351	203	0	1105	11	1319	0	7
96	217	0	1201	14	1432	0	7

mice of Table 1. Although mouse 12 was slightly exceptional, a clear correlation is evident between the numbers of mitoses and the numbers of large follicles produced. Divisions were generally most common in those ovaries containing the most large follicles. A similar phenomenon was apparent in the four ovaries of mice examined in the second period of post-ovulation oestrus, but here the connexion was between the numbers of mitoses and the numbers of follicles which burst. These counts are included in Table 3. Each animal had produced about the same total number of large follicles, but they varied in the numbers of these which had succeeded in shedding their eggs. Animals 96 and 351, in which all seven follicles had burst,

produced the greatest numbers of mitoses, and animal 85, in which two of the six follicles had failed to burst, showed the lowest number of mitoses. A further point of interest was that the large unburst follicles remaining in two of these animals had associated with them less mitoses per follicle than those of the mice in Table 2. In the second post-ovulation oestrous period each unburst large follicle had an average of about ten mitoses close to it, whereas in the pre-ovulation oestrous period each large follicle had an average of about double that number of associated mitoses.

There was also a tendency, noted in ovaries sectioned in all stages of the oestrous cycle, for the large growing follicles, or later the young corpora lutea, to be situated towards the anterior end of the ovary and consequently the mitoses of the germinal epithelial cells were also commonest in this region. The posterior part of the ovary, close to the fallopian tube, mainly contained older corpora lutea, and the germinal epithelium covering it showed little activity.

In metoestrus the young corpus luteum was fully formed, the reservoir of follicular fluid was usually absorbed, and the large luteinized cells showed few, if any, mitoses. In the four ovaries examined, the numbers of mitoses in the germinal epithelium varied considerably, the extremes being 49 and 128. Animals in early metoestrus had higher counts than those killed later in metoestrus, and as in both post-ovulation oestrous periods, the greatest number of cell divisions was in the region of the new corpora lutea. In the ovaries of all animals killed in metoestrus there were large numbers of very young oogonia immediately beneath the tunica albuginea.

The minimum average number of mitoses in the germinal epithelium was found in the first day of dioestrus, although as in metoestrus, there was considerable variation in the different animals between extremes of 9 and 50 divisions. The number of mitoses associated with the now developing crop of large new follicles was approximately equal to that connected with the newly formed corpora lutea, and this circumstance persisted into the second day of dioestrus. On this day also there was little change in the total number of mitoses, and in the four ovaries the extreme counts were 14 and 40. On the third day of dioestrus the average total number of mitoses was slightly greater, although still less than that recorded for pro-oestrus, and in the four ovaries examined, the counts varied between 19 and 43. These counts were made up largely of cell divisions occurring in the close proximity of the larger follicles, and the number of mitoses connected with the young corpora lutea was reduced. The cycle was completed with the merging of this condition into that already described for pro-oestrus.

DISCUSSION

The present observations on the ovary of the mouse fully endorse the statement made by Allen [1923] that new ova are produced throughout the active sexual life of the adult by a cyclical proliferation of the cells of the germinal epithelium. Allen noticed that during oestrus many more mitoses were present in the germinal epithelium, and consequently more ova were produced than in any other phase of the oestrous cycle, but he did not emphasize the fact that the maximum production of young eggs is confined to a very short post-ovulation period. The shortness and sharpness of this post ovulation proliferation of the germinal epithelial cells was described in the mouse and the starling, without the use of colchicine, by Bellerby & Gibbs [1941], and a further account of this phenomenon in the starling is to be

which usually has only one short breeding season annually, has been given by Bullough [1942*b*]. An exactly similar occurrence is also described [Bullough, 1942*a*] in the case of the annually breeding minnow in which, however, the post-ovulation period of activity of the cells of the germinal epithelium is of longer duration, due perhaps to the fact that very large numbers of new oogonia are produced.

As the connexion between the mitotic activity of the germinal epithelial cells and the oestrous periods or breeding seasons is so clearly marked, so also is the connexion in the mouse between the germinal epithelial mitoses and the large or recently burst follicles. By far the greatest number of mitoses are in regions closely adjacent to such follicles, and in those ovaries which contain fewer follicles than normal, or in which fewer follicles have succeeded in bursting, fewer mitoses are also evident. This connexion was not stressed by Allen [1923], who, in fact, stated that 'rapidly growing follicles and corpora lutea inhibit mitoses in the overlying epithelium'. His statement, however, was obviously only meant to refer to the tightly stretched squamous cells actually on top of the follicle, and it is confirmed here that these cells do not commonly undergo division. Allen did not mention the mitoses in the ring of cubical epithelial cells immediately surrounding each follicle or undeveloped corpus luteum, and it now appears that the cyclical nature of the production of new oogonia may well be directly due to the cyclical production of large follicles and consequently of developing corpora lutea. A similar association of mitoses of the germinal epithelial cells and large ovarian follicles or developing corpora lutea has been mentioned by Schmidt & Hoffman [1941] in the case of the guinea-pig.

Although other possibilities must also be borne in mind, the fact that the mitoses of the germinal epithelial cells are commonest at oestrus immediately suggests a possible connexion with the female sex hormone. This suggested connexion is strengthened by the observed association of the mitoses with large follicles which are full of follicular fluid rich in oestrogen. Further, when the follicle bursts, the liberated fluid, now actually bathing the adjacent germinal epithelial cells, may induce these cells to divide most actively and so to produce the very sudden and acute post-ovulation peak shown in the graph. As in the ferret [Robinson, 1918], the released follicular fluid may not be a mobile liquid but a viscous mass which, sticking in the immediate neighbourhood of the collapsed follicle, produces the results noted. Also, during the post-ovulation oestrous period when the corpus luteum is forming, follicular fluid is still being rapidly produced by the old follicle cells which have not yet become luteinized, and this action might be expected to reinforce any effect on mitosis started by the liberated follicular fluid. It might also be argued, however, that during this period the developing corpus luteum is producing some other hormone, perhaps even progesterone itself, which induces the observed post-ovulation mitosis peak, and it is also possible that the luteinizing hormone of the anterior pituitary gland is involved.

In spite of all the evidence [see review of Allen, Hisaw & Gardner, 1939] that the abnormal conditions set up by injections of oestrogen into adult female mammals inhibit follicle growth and cause sterility, it appears most probable that in the normal animal a high local concentration of female sex hormone is in some way connected with the mitoses of the germinal epithelial cells. It is also interesting to note that the only other cell divisions of oogenesis in the mouse, the meiosis and mitosis of

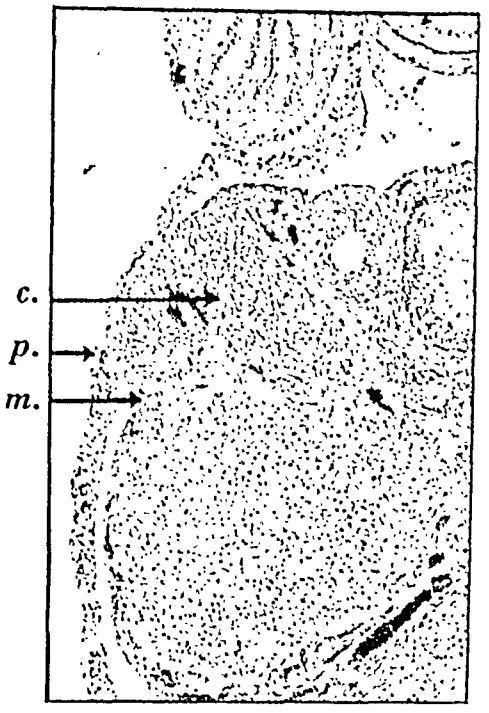


Fig. 3

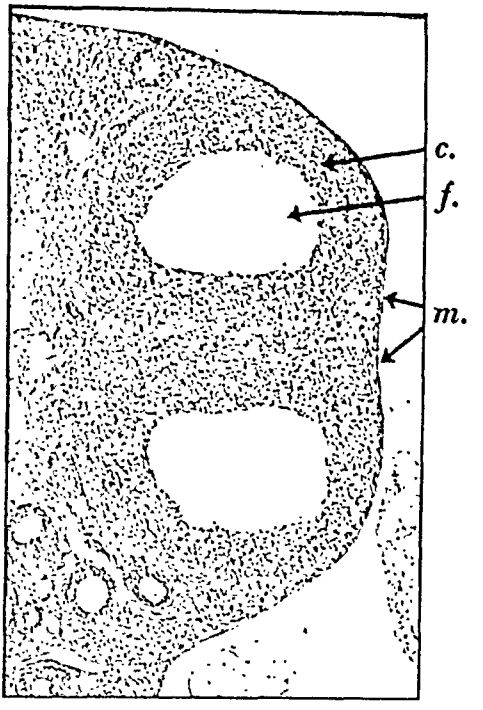


Fig. 4

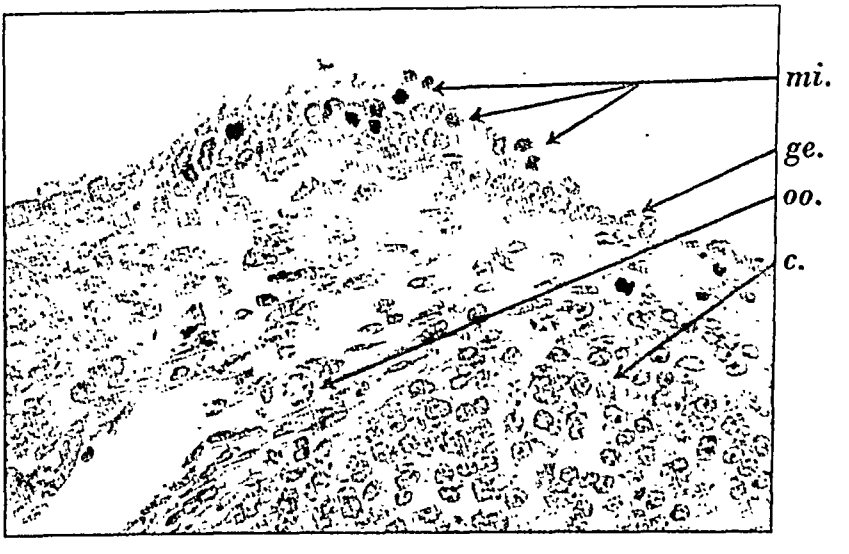


Fig. 5

the primary and secondary oocytes, take place at exactly the same time as the mitosis peak in the germinal epithelium, and it is possible that they too are connected with the female sex hormone. Further experimental work on this problem is required, and is now in progress.

SUMMARY

1. Counts were made of the total numbers of mitoses in the germinal epithelium of mouse ovaries in various phases of the oestrous cycle. It was found that mitotic activity was least in dioestrus, and that it rose slowly in pro-oestrus and pre-ovulation oestrus to reach a sudden high level in the short post-ovulation oestrous period. In metoestrus mitoses were again few.

2. By far the greatest number of germinal epithelial mitoses was found in the immediate neighbourhood of large follicles or undeveloped corpora lutea, and in those mice which produced less follicles than normal, or in which a smaller number than normal succeeded in bursting, less mitoses were evident.

3. The three divisions of oogenesis in the mouse, the mitosis of the germinal epithelial cell, the meiosis of the primary oocyte, and the mitosis of the secondary oocyte, all tend to take place in the same short post-ovulation oestrous period, and the possibility is discussed that their occurrence at this time is connected with the presence of high local concentrations of oestrogen.

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EXPLANATION OF PLATE 1

FIG. 3. Developing corpus luteum typical of the first post-ovulation oestrous period. The follicular fluid has escaped, and the old follicle cells hang out from the surface of the ovary. × 70.

FIG. 4. Developing corpora lutea typical of the second post-ovulation oestrous period. The germinal epithelium and tunica albuginea have reformed, and a new reservoir of tertiary follicular fluid is present in the centre of the cell mass. × 70.

FIG. 5. Group of mitoses among the germinal epithelial cells closely adjacent to a developing corpus luteum in the ovary of a mouse killed in the second post-ovulation oestrous period. × 400.

[illegible] developing corpus luteum [illegible] tertiary follicular fluid, [illegible] granulosa cells, [illegible] germinal epithelium, [illegible] tunica albuginea, [illegible] germinal epithelial cells, [illegible] the free [illegible] [illegible] [illegible]

THE METHOD OF GROWTH OF THE FOLLICLE AND CORPUS LUTEUM IN THE MOUSE OVARY

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(*Received 12 January 1942*)

A close association has been observed in the mouse ovary between the mitoses of the germinal epithelial cells and the large follicles or undeveloped corpora lutea, and the mitoses were found to be especially numerous at the times when the germinal epithelial cells were actually in contact with the follicular fluid [Bullough, 1942]. These facts have suggested the probability that the germinal epithelial cells are normally induced to divide by the close proximity of high local concentrations of follicular fluid rich in oestrogen, and it was considered possible, if this theory is correct, that the follicular fluid might also be a cause of division in other adjacent ovarian cells. In order to test this, the mitoses of the cells in the growing follicles and in the developing corpora lutea were closely studied. Except for the oocyte itself, these cells are situated nearest to the follicular fluid, and many of them are in direct contact with it.

MATERIAL AND METHODS

The mice used in these observations were the same as those used by Bullough [1942] in the study of the mitoses of the germinal epithelial cells, and for the details of experiment, reference may be made to that paper. It should be noted that, to obtain the maximum number of mitoses, the colchicine technique was used. The difficulties encountered by Lane & Davis [1939] in the use of this technique as a method for the study of follicle growth in rats were not experienced.

For the purposes of the investigation the ovarian follicles were classified into the following types.

(1) Small primary follicle. Slowly growing (up to about 100μ diameter) and lacking follicular fluid (Plate 1, fig. 2).

(2) Growing follicle. Rapidly enlarging (over about 175μ diameter) and with a single large reservoir of follicular fluid (Plate 1, fig. 2).

(3) Fully grown follicle. About 575μ in diameter and with very thin walls (Plate 1, fig. 3).

In each of these three types, many follicles of approximately equal size were closely examined, and all the mitoses in a hundred median sections were counted. The results are given as averages, together with standard deviations, of the numbers of mitoses present per section classified to show their relative abundance in the following zones (see Fig. 1).

(1) Membrana granulosa.

(*a*) *Oocyte zone.* In small primary follicles this included all cells inside a circle drawn with its centre at the centre of the oocyte and with a radius twice that of the oocyte. In the larger follicles it comprised the discus proligerus.

(*b*) Inner zone. Only present in the larger follicles, this included all cells within a line drawn through the follicle wall half-way between the inner edge, which is adjacent to the follicular fluid, and the outer edge, which is adjacent to the theca interna.

(*c*) Outer zone. Including all other cells.

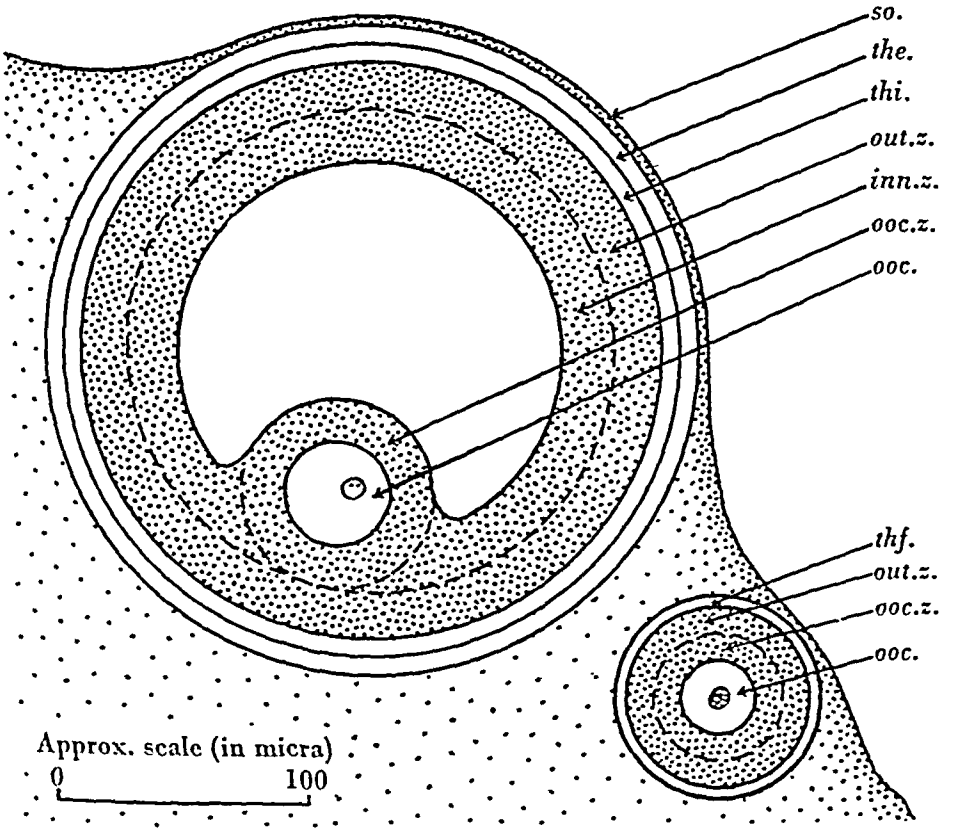


FIG. 1. Diagrammatic drawing of a small primary follicle and a larger rapidly growing follicle to show the positions of the various zones. *Inn.z.* inner zone of membrana granulosa; *ooc.* oocyte; *ooc.z.* oocyte zone or discus proligerus; *out.z.* outer zone of membrana granulosa; *so.* surface of ovary; *the.* theca externa; *thf.* theca folliculi; *thi.* theca interna.

(2) Theca folliculi. In the larger follicles this was subdivided into:

(*a*) theca interna;

(*b*) theca externa.

In the study of corpus luteum development, difficulties were encountered due to the shortness of the post-ovulation oestrous phase, and to the rapid and radical changes which took place during this period in the organization and mitotic activity of the mass of old follicle cells. It did not prove practicable to make a series of mitosis counts similar to those obtained for the follicle, and instead, only a description is given. This description is derived from the study of a series of twelve animals, no

OBSERVATIONS

Growth of the follicle

The follicles surrounding oogonia and very small oocytes lacked follicular fluid, and although many of them were constantly seen to be undergoing atresia, others appeared to grow slowly and steadily. The mitosis counts obtained for a group of these primary follicles, which had walls three or four cells in thickness and an approximate total diameter of 80 μ, are shown in Table 1. In view of the large standard deviations there were not great differences between the counts in the different zones. However, the theca contained the lowest average number of mitoses and the oocyte zone the largest, and as the oocyte zone also presented the smallest area, there were clearly signs of a gradient of mitotic activity greatest near the oocyte and least in the theca.

Table 1. *Average numbers of mitoses per section in the various zones of the follicle wall*

Follicle			Numbers of mitoses					
			Membrana granulosa			Theca		
Type	Diam.	No. n*	Oocyte ar. $\pm \sigma$*	Inner ar. $\pm \sigma$	Outer ar. $\pm \sigma$	Folliculi ar. $\pm \sigma$	Interna ar. $\pm \sigma$	Externa ar. $\pm \sigma$
1	80 μ	100	1·33 ± 1·15	—	0·98 ± 1·04	0·60 ± 0·75	—	—
2	350 μ	100	28·41 ± 4·01	65·05 ± 6·59	7·86 ± 2·88	—	4·51 ± 2·13	1·21 ± 0·96
3	575 μ	100	31·96 ± 4·53	7·29 ± 2·44	1·56 ± 1·58	—	1·60 ± 1·09	0·61 ± 0·66

* ar. = arithmetic mean of the number of mitoses, σ = standard deviation = $\sqrt{\frac{fd^2}{n}}$, where n = number of median sections of follicles examined.

When the primary oocytes reached an approximate diameter of 100 μ, follicular fluid suddenly appeared in several places simultaneously, and immediately the follicle growth was greatly accelerated. The early stages of this rapid growth phase were not examined in detail owing to the difficulty, when so many separate pools of follicular fluid were present, of separating the cells into clearly defined zones. Each of the small rapidly growing follicles appeared to persist for at least one oestrous cycle, and large numbers of them were continually ceasing to grow, disorganizing, and disappearing. The next counts were therefore made on the fewer larger follicles which had passed this danger period. The follicles studied had approximate diameters of 350 μ, and they were found to be especially common in late dioestrus and in pro-oestrus. They were all undergoing extremely rapid growth, and the average numbers of mitoses present are shown in Table 1. A steep gradient of mitotic activity, highest near the oocyte and the follicular fluid and lowest in the theca externa, is immediately obvious. In relation to its size, the discus proligerus, or oocyte zone, showed the greatest number of cell divisions, and the inner zone of the membrana granulosa, near the follicular fluid, was also extremely active (Plate 1, fig. 2). Many of the mitoses in the inner zone of the membrana granulosa, and the majority of those in the outer zone, were situated at the sides and base of the discus proligerus, and mitoses were uncommon in the larger area of the outer zone not adjacent to the discus proligerus. The theca interna showed less activity than the outer zone of the membrana granulosa, but the mitoses were evenly distributed. Divisions of the cells of the theca externa were relatively uncommon.

The speed of growth of these large follicles slowed during the pre-ovulation oestrous period, and when a diameter of between 550 and 600 μ was reached, mitotic activity was considerably reduced. Except in the region of the discus proligerus, all layers of the follicle wall were thinner. The mitosis counts of the different zones of such follicles are shown in the table, and it is seen, especially in the inner zone of the membrana granulosa, how relatively few were the cell divisions present. Only the cells of the discus proligerus, or oocyte zone, were more active than before, and almost all the mitoses recorded as present in the inner and outer zones of the membrana granulosa were in regions closely adjacent to the sides of the discus (Plate 1, fig. 3). The granulosa cells in these regions were often loosely separated by small pools of follicular fluid, and it appeared that an active secretion was taking place into these areas. Mitoses were rare in all parts of the membrana granulosa not in the close proximity of the discus proligerus, and in the inner zone near the follicular fluid necrotic cells were numerous. The cells of the thecae interna and externa also showed fewer divisions than before, but those which were present were evenly distributed.

Growth of the corpus luteum

Immediately after ovulation had taken place, the tunica albuginea and germinal epithelium were torn, and the cells of the old membrana granulosa protruded from the ovary surface [see Bullough, 1942]. Divisions were common among all the cells of the young corpus luteum, and an average of about thirty mitoses was to be seen in a single median section. After an hour or two, mitoses became rare in the centre of the cell mass, although they remained common among the cells which protruded from the ovary surface and were especially so in the extreme outer layer. The protruding cells were slowly retracted into the ovary, and about 6 hr. after ovulation the tunica albuginea and germinal epithelium were reformed over the young corpus luteum. The cells which had protruded now showed little mitotic activity, and they came to lie on the outside of a cell mass in the centre of which a new reservoir of follicular fluid was formed. As in the growing follicle, mitoses were now seen among the cells of an inner zone adjacent to the follicular fluid, but the mitotic activity was much weaker than before ovulation. When the reservoir of follicular fluid was newly formed, there was an average per section of about fifteen mitoses in the inner zone of the wall, whereas in the outer zone there were usually only two or three mitoses. The numbers of divisions fell rapidly, however, and when early metoestrus was reached, the reservoir of follicular fluid was in process of absorption, the old granulosa cells were becoming luteinized, and no mitoses were to be found anywhere.

DISCUSSION

It has been noted by Engle [1931], in a description of the development of the ovarian follicles of young rats, that the time of the first appearance of follicular fluid marks the beginning of a period of rapid follicle growth. It is now shown that the growth of the young primary follicle in the adult mouse also proceeds slowly until, suddenly, when the follicular fluid begins to appear, the growth rate is greatly accelerated. Further, in the resulting rapidly growing follicle a gradient of mitotic activity, highest near the follicular fluid, is evident throughout the thickness of the follicle

wall, and although mitoses become less numerous when the follicle is approaching full size, a similar gradient is still evident.

The reason for the slackening of mitotic activity in almost fully grown follicles is not obvious. It is possible that the cells, becoming in some way exhausted, are unable to divide further, and it should be noted that at this time some necrotic cells make their appearance in that part of the membrana granulosa nearest the follicular fluid. It is also possible that, in the later stages of follicle development, the secretion of follicular fluid takes place so rapidly that growth by mitosis is no longer able to keep pace with the rate of follicle expansion, and that this results in such a tension in the follicle wall that mitosis is slowed down. Mechanical stretching of the germinal epithelial cells is known to hinder their division [Allen, 1923]. It is only in the discus proligerus and the adjacent membrana granulosa that there is little or no tension, and in these regions mitosis continues unabated. Finally, it is also possible that the secondary liquor folliculi, which is probably being rapidly formed at this time [Robinson, 1918], is in some way connected with this change in the abundance of mitoses, or again, some combination of all of the above factors may be responsible.

In each of the three types of follicle examined, it was noted that the cells nearest to the oocyte were undergoing the most active division. Even in the small primary follicle mitoses were commonest among the cells of the oocyte zone, but the effect was greatest in the discus proligerus of the rapidly growing and fully developed follicles. In these larger follicles the effect is probably a further expression of the observed connexion between mitoses and follicular fluid. The discus proligerus projects into the reservoir of follicular fluid exposing to it a relatively great surface area, and further, as already noted, in the fully developed follicle it appears to be the only part not under tension. However, it has been observed that the mitoses in the remainder of the membrana granulosa are more common in regions adjacent to the discus proligerus than they are elsewhere, and many of these dividing cells, especially in the outer zone, are at a relatively great distance from the follicular fluid. The possibility therefore remains that the mitoses of the oocyte zone are, in some way, at least partly associated with the oocyte itself.

When tension is suddenly released at ovulation, a wave of cell division occurs which affects all parts of the young corpus luteum. This activity quickly dies down, perhaps due to the absence of large quantities of follicular fluid inside the cell mass, and only the extreme outer layer of cells continues to produce numbers of mitotic figures. This last phenomenon is directly comparable with the burst of mitotic activity in those germinal epithelial cells adjacent to the young corpus luteum. Bullough [1942] has described this as being probably due to the bathing of the germinal epithelial cells in the viscous follicular fluid which, extruded from the burst follicle, still remains in the periovarian space. As this effect dies down, a fresh activity begins among the cells adjacent to the newly forming reservoir of tertiary follicular fluid.

It is thus apparent that the divisions of the cells of all layers of the follicle wall, as well as those of the cells of the young corpus luteum, bear a clear relation to the close presence of liquor folliculi which is known to be rich in oestrogen. This relation is exactly similar to that already described by Bullough [1942] for the cells of the

germinal epithelium. Although experimental proof is still wanting, it appears, in the normal mouse, that oestrogen not only causes mitoses of the cells and growth of the organs of the female accessory sexual system, but that it also induces the same effects inside the ovary itself. Further, if this proves to be the case, it is clear that the ovarian cells require a far higher concentration of oestrogen in their immediate neighbourhood before they are induced to divide than do the cells of the accessory sexual organs. The present results, together with those of Bullough [1942], will, if they are confirmed, make necessary a reorientation of view concerning the endocrine mechanism of the ovary.

SUMMARY

1. A description is given of the zoning of cell divisions in the growing follicles and corpora lutea of the mouse ovary.

2. It is shown that mitosis is most common in those follicle cells closest to the oocyte and to the reservoir of follicular fluid, and that a gradient of mitotic activity exists throughout the thickness of the wall with its lowest point in the theca externa.

3. In the developing corpus luteum mitoses are at first most frequent in the old membrana granulosa cells nearest to the extruded follicular fluid, but later they become common among cells close to the newly formed reservoir of tertiary follicular fluid.

4. It is concluded that the oestrogen, known to be present in a high concentration in the follicular fluid, not only induces mitosis and growth in the accessory sexual organs, but also, in all probability, in the ovarian cells themselves.

Addendum

A further survey of the ovaries on which the present paper is based has indicated the probability that cells other than those of the follicle and corpus luteum are affected by the close presence of high concentrations of female sex hormone in the follicular fluid. The connective tissue cells and the cells of the ovarian stroma were found to pass through a cycle of mitotic activity which could be correlated with the cyclical growth of the follicles. Very few divisions of these cells were seen during the early part of dioestrus, but many were present in the ovaries of mice killed in pro-oestrus, when the follicles were rapidly growing, and in the pre-ovulation oestrous period, when the follicles were fully developed. Further, at the time of maximum activity, mitoses were most frequent among cells close to large follicles, and least frequent among cells close to corpora lutea. At all times the divisions of the connective tissue cells and the cells of the ovarian stroma were rarer than those of the cells of the outer layers of the follicle wall.

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EXPLANATION OF PLATE 1

FIG. 2. Sections of a small primary follicle entering the transition stage and of a rapidly growing follicle with a single reservoir of liquor folliculi. In the larger follicle mitoses are common in the discus proligerus and the inner zone of the membrana granulosa. × 220.

FIG. 3. Section of a fully grown follicle showing the thin wall. The greater part of the membrana granulosa is devoid of mitoses, but these are still common in the discus proligerus and in the zones closely adjacent to it. Note the small pools of liquor folliculi among the cells at the sides of the discus proligerus. × 220.

dp. discus proligerus; *f.* pool of follicular fluid; *inn.* inner zone of the membrana granulosa; *lf.* reservoir of follicular fluid; *mg.* membrana granulosa; *o.*1. oocyte in a small primary follicle; *o.*2. oocyte in a rapidly growing follicle; *o.*3. oocyte in a fully grown follicle; *out.* outer zone of the membrana granulosa; *th.* theca.

Photomicrographs by Mr J. Manby, F.R.P.S., University of Leeds.

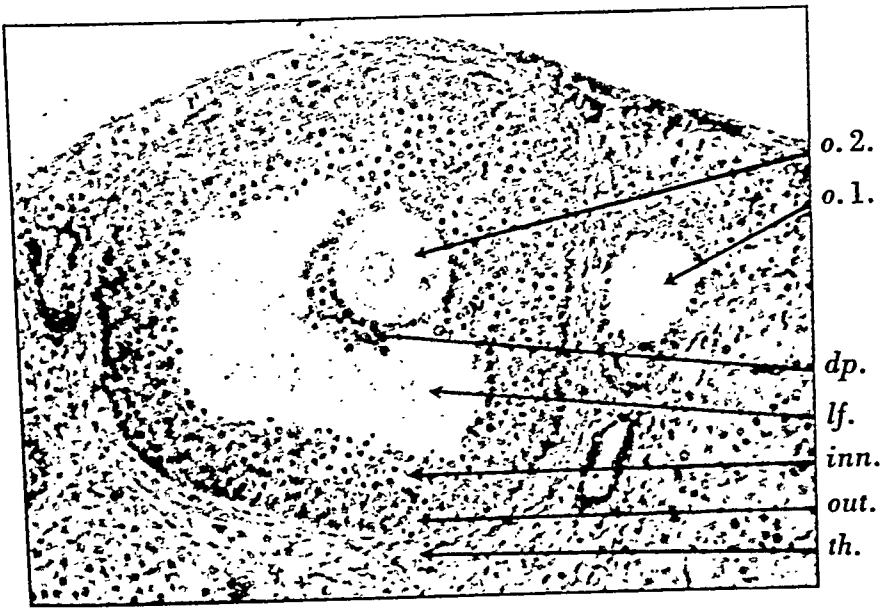


FIG. 2

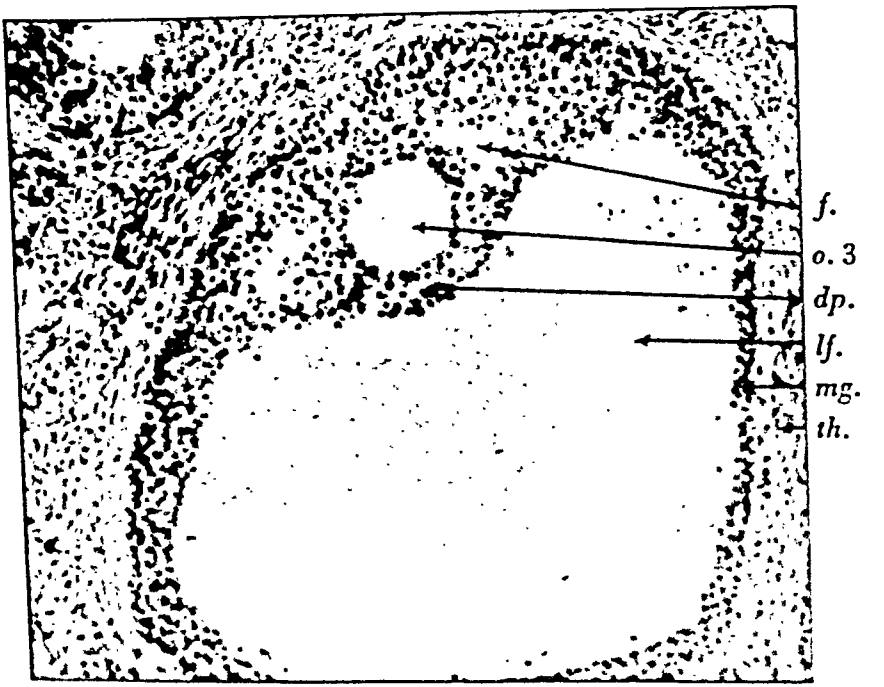


FIG. 3

ACTIONS OF SEX HORMONES ON OESTROUS CYCLE AND REPRODUCTION OF THE GOLDEN HAMSTER

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(*Received 19 January 1942*)

Some biological features described by Bruce & Hindle [1934] and by Deanesly [1938] suggest that the golden hamster might be a good subject for the study of sex hormones. For instance, the animal usually stops breeding at the very early age of 10 months, though it may attain an age of at least 2 years; and there are generally only three litters, though the period of gestation (16 days) is very short. As a rule the animals do not breed in the winter, though according to Deanesly [1938] the oestrous cycle continues regularly throughout the whole year.

A detailed account of the histology of the ovaries and secondary sex organs, during the oestrous cycle and pregnancy, has been given by Deanesly [1938]. The action of oestrone and of progesterone on the vaginal mucosa in castrates has been studied by Klein [1937], who later [1938] showed the necessity of the corpus luteum for the maintenance of pregnancy.

These investigations were all carried out on animals from Professor Hindle's stock descended from the litter of golden hamsters found in 1930 in the neighbourhood of Aleppo, Syria [Aharoni, 1932], and first bred at the Hebrew University, Jerusalem. The following year Dr S. Adler presented two pairs descended from the original litter to Professor Hindle, who succeeded in raising a stock of hamsters which have been distributed to various laboratories and form the basis of all observations in this country in which these animals have been used.

In this stock several females were found either to have been sterile during their life cycles, or to have ceased breeding long before the age of 10 months. Certain features observed by Hindle support the supposition that this sterility, at least in part, might be the result of genetic factors. During the past breeding season experiments have been made on the sexual activation and reactivation of these sterile animals by means of oestrogens, applying the findings of Steinach, Heinlein & Wiesner [1925], Steinach, Stäheli & Grüter [1934] and Stäheli [1935] on the effect of these substances in rats, guinea-pigs and cows, or by means of gonadotrophic extracts known to be active in rodents [Zondek & Aschheim, 1927; Smith & Engle, 1927; Steinach & Kun, 1928] and sheep [Parkes & Hammond, 1940]. At the outset, however, it was necessary to investigate the action of various amounts of these hormones on the normal oestrous cycle, in order to avoid doses which might inhibit the regulatory activities of the pituitary [Zondek, 1937] instead of stimulating them. This paper contains the results of these last-mentioned experiments as well as those of the first few experiments on activation and reactivation of sterile females.

METHODS

The experiments were carried out during the breeding season (March–October) with animals maintained under the same conditions as regards feeding and care as for some years previously.

At first the vaginal smears were examined over a period of at least four oestrous cycles, and fertility or sterility established by mating on three occasions with potent males. With a few exceptions, indicated below, the females were left with the males for the duration of two to three oestrous cycles, but never longer than 14 days, and then isolated.

The vaginal smears were taken from the upper vagina by means of a platinum loop. A plug-like mass of cornified scales, ultimately stopping the vulva, was sometimes found in the lower part of the vagina, and had to be removed before the insertion of the platinum loop. The smears were fixed and stained either by Leishman's method or with Ehrlich's haematoxylin, after fixing in methyl alcohol for at least an hour. Sections of the ovaries and uterus were stained with Ehrlich's haematoxylin and eosin. The organs were weighed after fixing in Bouin's solution and transferring to 70% alcohol as described by Deanesly [1935].

Each dose of oestrogen used for injection was dissolved in 0·1–0·2 ml. of arachis oil. The following gonadotrophic extracts were used: chorionic gonadotrophin (Pregnyl, 1 i.u. in 6·6 μg.; or Antroidin), a mixed horse anterior pituitary extract (AP118B), and acetone-desiccated old mare anterior pituitary (OMAP), each dose being dissolved in 0·2–0·4 ml. of water. The solution of AP118B and OMAP was carried out according to Parkes's directions. The powder was well triturated with water added drop by drop, then dissolved by the addition of a few drops of dilute NaOH and the solution neutralized with dilute acetic acid.

THE OESTROUS CYCLE

As Deanesly has already shown, a sticky discharge, rich in epithelium, may be squeezed from the vulva every 4 days, at the time of ovulation. At this stage the smears consist of a dense agglomeration of predominantly round or oval epithelial cells, which in form and size are similar to those which in the Chinese hamster [Parkes, 1931], rat and mouse indicate pro-oestrus, and in the ferret [Hamilton & Gould, 1940] the first stage of oestrus. Among these were also seen columnar epithelial cells, pale or basophil scales and sometimes isolated leucocytes. On the days these smears were taken, the ovaries already contained fresh corpora lutea and the uteri were congested and enlarged (178–231 mg.). Within about 24 hr., the smears show invasion by a mass of leucocytes and the scales also increase in numbers. This richness in leucocytes, which is seen immediately after oestrus, in all probability indicates metoestrus. However, leucocyte smears were found, not only in metoestrus, but also intermittently, or even continuously, in castrated hamsters, and also in those which had no oestrous cycle and were sterile. But whereas in the latter the uterus was pale and thin, it was congested and enlarged in hamsters during metoestrus. When epithelial smears were found the females were in heat. This is shown by the fact that parturition occurred exactly 16 days after the day on which the last epithelial smear was observed, and in addition only those females which had been mated on the 2 or 3 days when an epithelial smear was seen, produced litters. Also, in the case of certain individuals showing epithelial smears as a result of the injection of 200 i.u. of chorionic gonadotrophin, mating was observed even during the morning, although normally this only occurs during the night.

On the third and fourth day after the beginning of the oestrus, epithelial cells, leucocytes and scales decreased in numbers, and sometimes the smears showed only coagulated fluid and a few scales. At this stage the uterus was not congested and not enlarged (71–120 mg.).

Regular observation of the vaginal smears and of the shape of the ovaries and of the uterus resulted in four groups being distinguished:

Group 1. Twenty-seven animals which showed regular 4-day cycles and were fertile. In sixteen of these, aged between 2 and 10 months, the litters were viable and fertile. The litter of the 17th, a 16-months-old female, died immediately after birth.

Group 2. Ten animals, aged 5–15 months, in which oestrus occurred at intervals of 2–6 days. Of these, four were younger than 10 months and bred in spite of their irregular cycles; two, 6 months old, were sterile. Four, 10½–16 months old, were sterile.

Group 3. Twenty-nine animals, aged 7–21 months, showing regular 4-day cycles, but which were without exception sterile.

Group 4. Ten animals, aged 3½–23 months, in which oestrus never occurred, or only very seldom (e.g. once in 34 days), were also sterile.

ACTION OF OESTROGENS

Small and moderate doses

Ten animals received single injections of 1, 2 or 40 μg. of stilboestrol, and five of 1 or 2 μg. of oestradiol benzoate. All the animals belonged to groups 1 and 3 aged up to 14 months. Among these fifteen animals, the oestrous cycle was unaffected in two treated with 1 and 40 μg. stilboestrol. In all the other individuals, oestrous smears appeared within 40–48 hr. after the injection. Those injected with doses of 1 or 2 μg. of either substance, showed the characteristic smears for less than 24 hr., whilst in those receiving a dose of 40 μg. of stilboestrol, these smears persisted for 2–5 days (Plate 1, fig. 1). Spontaneous oestrus appeared 4–8 days after the end of the last artificially produced oestrus, and the cycles continued regularly.

Three more females from group 3, aged from 10 to 14 months, were mated, 2–4 weeks after injection of 40 μg. of stilboestrol, with the same males that had failed to fertilize them before the injection. Two of them produced litters while the third remained sterile.

In four females of group 4, aged 3½–22 months, single injections of 40 μg. of stilboestrol also provoked oestrous smears, which began, on an average, 40 hr. after the injection and persisted for 3 days. In two of these animals (aged 11 and 19 months) regular cycles were established 7 and 9 days respectively after the expiry of the period of provoked oestrus. 18 days after the injection the animals were successfully mated. As regards the other two females, in the old one (22 months) the effect concluded with three regular 4-day cycles, while in the young one (3½ months) a spontaneous oestrus occurred 11 days after expiry of the reaction, but did not recur. Both animals remained sterile.

The litters provoked by the oestrogen died within 2–4 days. One of the mothers in group 4 (19 months old) was reinjected 1 month after parturition, and as in the first experiment, mated 18 days after injection with the same male. It littered again, and of the seven young, one survived and proved to be fertile.

Large doses

Three groups each of five animals (ten from group 3, aged 8–19 months, and five virgin females with regular 4-day cycles, aged 3 months) were injected daily with 200 μg. of stilboestrol, oestrone and oestradiol benzoate, respectively, for 6 consecutive days. The three oestrogens produced in all the animals a characteristic alternation of periods of oestrous smears with periods of leucocyte smears. The epithelial periods persisted for 1–7 days after oestradiol benzoate, 1–10 days after stilboestrol and 2–6 days after oestrone. The leucocyte periods lasted up to 4 days, and during this period the uterus, examined by laparotomy, was enlarged and greatly expanded. The phenomenon began, on an average, 40 hr. after the first injection and persisted, in animals injected with oestradiol benzoate for 25–26 days, with stilboestrol 9–19 days, and with oestrone 10–12 days.

Six animals castrated either before or after puberty, which received 200 μg. of oestradiol benzoate for 6 consecutive days gave different smears from normal females receiving the same dose. After 40 hr., when in all probability only a small quantity of the injected hormone had been absorbed, epithelial smears appeared. Subsequently, the epithelial cells disappeared almost completely and the smears contained mainly slightly acidophil, basophil or pale cornified scales. These cornified smears alternated at irregular intervals with leucocyte smears. Shortly before the disappearance of the reaction, that is to say when the greater part of the hormone had already been excreted, the number of cornified scales decreased and the number of epithelial cells again increased. This experiment was repeated with this series of animals with similar results using the same dose of oestrone instead of oestradiol benzoate.

A somewhat similar effect was observed in three additional uncastrated animals after injection of enormous quantities (five injections of 1–3 mg. of oestradiol benzoate). From time to time, during the peak of the oestrogenic action, smears occurred in which the cornified scales considerably exceeded the epithelial cells in number.

Three of the original group of fifteen hamsters which had been injected with six doses of 200 μg. of oestrogen were killed for histological examination. In eleven out of the twelve survivors, spontaneous oestrus occurred as early as 3–8 days after the last oestrous smears, and was followed by regular 4-day cycles. In only one individual (oestradiol benzoate, group 3, 19 months old) the oestrous cycle failed to return, and there was a marked progression of senile symptoms such as falling out of the hair, kyphosis of the lumbar vertebral column, and decrease in tonicity of the vulva. Nine of this group including the three virgin oestrone-treated animals, were mated 8–24 days after the disappearance of the oestrogenic effect. These three females littered; the remainder have been twice mated but have remained sterile.

ACTION OF GONADOTROPHIC EXTRACTS

Effect in immature females

The gonadotrophic effect of AP118B and of chorionic gonadotrophin in immature females was tested using five animals for each experiment. Dose-response curves could not be drawn owing to the lack of sufficient numbers of animals.

In the 100 hr. test, AP118B was divided into three equal doses injected on 3 successive days; chorionic gonadotrophin was given in a single injection [Evans & Simpson, 1929].



Fig. 1. Vaginal smear of a non-castrated golden hamster 68 hr. after an injection of 40 μg. of stilboestrol.



Fig. 2. Vaginal smear of a [illegible] hamster treated with 200 i.u. of chorionic gonadotrophin daily on 5 consecutive days.

In the weight test [Deanesly, 1935] both extracts were divided into equal doses given on 5 successive days.

The effect of both gonadotrophins seems to be less in immature female hamsters than in immature rats, and to be in a high degree dependent on the age of the animals. 4 mg. of AP118B, for example, brought about an increase of the ovary weight from 13 to 25·7 mg. in 24-day-old hamsters weighing from 25 to 30 g., while in the immature rat a dose of 1·5 mg. trebles the ovary weight [Parkes, personal communication].

4 mg. of AP118B, during 100 hr. tests, produced in 24-day-old hamsters oestrous smears, ripe follicles and luteinization; but in those 18 days old, oestrous smears without ripe follicles.

80 μg. (12 i.u.) of chorionic gonadotrophin produced oestrous smears in 24-day-old but not in 18-day-old females, while a dose of 160 μg. was effective in both groups. The oestrous smears began 90–96 hr. after injection and disappeared in less than 24 hr.

If one may judge from a few preliminary experiments, females aged 30 days appear to react with greater sensitivity to both extracts. These animals, however, were already at or near puberty, since the first spontaneous oestrus, as was shown by the controls, may occur at as early an age as 32 days.

Progressive application of gonadotrophic extracts produced characteristic changes in the vaginal smears. Five 24-day-old females received 4 mg. of AP118B daily for 12 days; five more, of equal age, received 1320 μg. (200 i.u.) of chorionic gonadotrophin daily. The two extracts were given in the same relative quantities as those which produced approximately equal increases of the ovary weight. 96 hr. after the first injection, oestrous smears appeared. After 7–10 days the vaginal smears changed their character and then consisted of a strikingly thin, slimy fluid, and could no longer be fixed by Leishman's method. After fixing with methyl alcohol and staining with Ehrlich's haematoxylin very small round cells, cell debris and scattered nuclei were seen, as well as columnar epithelial cells with small nuclei.

These columnar cells were similar in shape to mucifying epithelial cells, such as Deanesly found in the upper vagina of pregnant hamsters, and Klein in hamsters treated with oestrogen and progesterone, but I was unable to obtain any mucicarmine reaction. The smears contained most of these cells 9–13 days after the first injection. After this the secretion of the thin slimy fluid increased, while the columnar cells gradually diminished in size and number and seemed to disintegrate more readily.

The production of these typical vaginal smears was accompanied by another phenomenon. In the AP118B-injected animals the clitoris became markedly congested and enlarged from 8 to 11 days after the first injection. In the animals injected with chorionic gonadotrophin this was observed rather sooner—in one of them only 70 hr. after the first injection. While the hyperaemia, the pacemaker of the hormone action [Steinach, Kun & Peczenik, 1936], wore off in the course of the next few days, the enlargement of the clitoris markedly increased.

Large doses of oestrogen (injections of 200 μg. of stilboestrol on 5 successive days), which are known to produce vasodilatation of many vascular regions, likewise produced hyperaemia and enlargement of the clitoris in immature animals. The enlargement was, however, evidently less, both in degree and in duration, than that following the injection of gonadotrophic extracts. In the spontaneous oestrus of adult females

the clitoris was not significantly congested or enlarged. In the AP118B-injected animals the ovaries contained ripe follicles, massive corpora lutea and luteinized follicles; the uteri showed a slight but clear luteal phase. In the ovaries of the animals treated with chorionic gonadotrophin the follicles were strikingly small. Several follicles and the greater part of the stroma were luteinized.

Effect in adult females

Effect of moderate doses

Five animals from groups 1 and 3 each received single injections of 8 or 24 i.u. of chorionic gonadotrophin; another five from the same groups, daily injections of 2 mg. of AP118B on 3 successive days. Oestrus occurred on the average 64 hr. after either the single injection of chorionic gonadotrophin, or the first of the three injections of AP118B, and lasted up to 4 days independently of the stage of the oestrous cycle. This is earlier than the start of oestrus in immature hamsters which usually takes 96 hr. In the animals treated with chorionic gonadotrophin the regular oestrous cycle was resumed 4–8 days after the cessation of the provoked oestrus; in those treated with AP118B the cycle was resumed as early as 4–5 days after the last injection.

Six further animals from group 3, of which four had received 20–24 i.u. and two 200 i.u. of chorionic gonadotrophin, in single injections, were mated after the onset of provoked oestrus. Three of them (two aged 7 months, the third 22 months) became pregnant. The other three, i.e. two 11-months-old hamsters injected with 200 i.u., and one 7-months-old female with the smaller dose, remained sterile.

Four animals from group 4 were mated after single injections of chorionic gonadotrophin (16–24 i.u.). In three of them mating took place after the onset of provoked oestrus. In the fourth, a 4-day cycle was recorded after the provoked oestrus had ceased, and it was mated during spontaneous oestrus. The last, as well as its two sisters, all aged 7 months, produced litters, but the fourth, aged 24 months, remained sterile.

In a fifth animal from this group (7 months), oestrous cycles lasting 4–6 days developed after injection of 24 i.u. of chorionic gonadotrophin. 19 days after this injection a further injection of 10 i.u. was given and the animal was successfully mated 11 days later.

While the activated females under 10 months old from groups 3 and 4 produced up to three litters each, which survived and proved fertile, the old activated female from group 3 littered only once, and her young died within the first 4 days after birth.

In the four activated females from group 4, regular oestrous cycles were recorded throughout the breeding season, apart from the anoestrus caused by pregnancy and lactation. The fifth animal, however, which had remained sterile, exhibited only a single oestrous cycle, and its ovaries were found to contain large cystic follicles.

Effect of large doses

One group of five adult animals received injections of 200 i.u. of chorionic gonadotrophin on 5 consecutive days, and another similar group 4 mg. of AP118B on 10 consecutive days. As early as 3–5 days later, shortly after the onset of provoked oestrus, columnar-cell smears appeared. In one of the former group these smears

even appeared without the previous occurrence of oestrous smears. Columnar cells were especially numerous and well formed in the earliest smears (Plate 1, fig. 2) while, subsequently, as in immature animals, they rapidly decreased in number, size and state of preservation with the increase of mucous secretion. Among them were seen isolated, larger, plate-like epithelial cells, also small cells of the oestrous epithelial type. The latter decreased within the first week in the animals injected with chorionic gonadotrophin, but in the AP118B-treated animals, not appreciably before the second week after the first injection.

In five more females, which had been injected daily with 5 mg. of OMAP on 10 consecutive days, the columnar cells first appeared 4–7 days after the first injection. The oestrous epithelium did not seem to decrease regularly, as in the animals which had received the other extracts, but to vary irregularly or to show an actual increase after a short period of decrease.

Effect of succession of moderate doses

Even smaller quantities of anterior pituitary principle, when sufficiently spread over a period of time, appear to produce columnar-cell smears. These were also seen in three animals to which a total dose of 10 mg. of AP118B had been given, spread over 5 days. A single injection of 10 mg. of AP118B, however, or five injections of 1 mg. did produce oestrous smears but no columnar-cell smears. Among three further animals (two from group 3, aged 4 and 11 months; one from group 4, aged 22 months) which had received daily injections of 2 mg. of OMAP on 5 successive days only one showed columnar-cell smears. The other two showed only oestrous smears lasting for 4 days.

None of the animals receiving moderate amounts of anterior pituitary gonadotrophin showed any lasting disturbance of the oestrous cycles. 4–7 days (15 days in only one case) after the last columnar-cell smears or oestrous smears, spontaneous oestrus in regular cycles was re-established.

Of the animals, on the other hand, which, in the course of 5 or 10 days had received relatively large quantities of gonadotrophin, the hamsters receiving chorionic gonadotrophin behaved differently from those which had received anterior pituitary hormone. In the first case spontaneous oestrus recurred 4–9 days after cessation of the reaction and was followed by regular oestrous cycles: in the second, no oestrous cycle reappeared. With the exception of two individuals which showed oestrus once and three times respectively during 2 months, all remained completely anoestrous and showed continuous or irregularly periodic leucocyte smears.

Forms similar to the cells shown in Plate 1, fig. 2 were often found shortly before the beginning of oestrus, both in physiological oestrus and in that provoked by gonadotrophin. Except in the case of two individuals, however, they were not found in animals whose oestrus had been caused by oestrogens. They were, on the other hand, found in pregnant animals, especially in the second week of pregnancy.

Their occurrence in eight non-castrated animals from group 3 (6–14 months old), which had received six daily injections of 0·25 or 0·40 mg. of progesterone on 6 successive days, is in agreement with this observation. In this experiment, the typical smears appeared, on an average, 4 days after the first injection. As early as 7 days, on an average, after the last injection, anoestrus came to an end with an oestrus

lasting 2–3 days. This oestrus was never obtained in castrated animals treated in the same way and, therefore, cannot be the result of an oestrogenic effect of the progesterone.

DISCUSSION

The results show certain peculiar features in which the golden hamster, in its reaction towards sex hormones, differs not only from other species but also from other rodents. In the first place mention must be made of the marked difference which exists between the actions of the same dose of oestrogen on normal and castrated females. In the former, the oestrogen produces a vaginal smear substantially the same as that shown in physiological oestrus; it resembles the vaginal smears given by rats and mice, both in pro-oestrus and, exceptionally, in metoestrus, and by ferrets [Hamilton & Gould, 1940] at the beginning of oestrus.

In castrates, the oestrous smears typical of hamsters were found only at the beginning and near the end of the reaction; the smears produced at the height of absorption were substantially the same as oestrous smears in rats and mice.

Klein, in castrated hamsters, found that very small amounts of oestrogen produced proliferation of the mucosa of the upper vagina and only slightly larger doses produced cornification. A comparison of our results with those of Klein shows that the dose of oestrogen which produces cornification in castrates produces vaginal smears in non-castrates corresponding to the condition of the mucosa produced in castrates by $\frac{1}{8}$–$\frac{1}{40}$ of this dosage.

This result permits of various interpretations: either the oestrogens are very much weakened in their action in non-castrates; or the vaginal mucosa of the non-castrates is less sensitive; or the absorption of oestrogens proceeds much more slowly than in castrates.

The last assumption would agree with our present knowledge of the relation between the rate of absorption and the action of hormones [Deanesly, 1939]. Against this is the fact that the quickly absorbed oestrone produced smears identical with those of the slowly absorbed oestradiol ester. Comparative estimations of the absorption of oestrogen tablets [Deanesly & Parkes, 1938] in castrated and non-castrated hamsters, respectively, may contribute to the clarification of this question.

Periodic changes in the vaginal smears, due to oestrogens, seem to be more readily produced in golden hamsters than in other rodents. Kun [1935] sometimes found them in castrated rats which had received 8600 m.u. of oestrone benzoate, distributed in twenty-four daily doses. Emmens [1939] observed them after implantation of oestrone crystals and with injections of esterified, but not of free, stilboestrol. I have not observed this phenomenon in rats with either stilboestrol or oestradiol benzoate.

Zuckerman [1938] observed 'artificial oestrous cycles' in castrated rats during prolonged treatment with small quantities of oestrone, but only within a very limited range of dosage. Zuckerman's experiments make it very probable that the adrenal cortex produces cyclical changes in the sensitivity of the rat to oestrogenic stimulation, and suggest that oestrus itself may be weakened at the time when leucocyte smears appear. Against the latter assumption stand recent experiments of Bourne & Zuckerman [1941] and the above-mentioned experiments of Kun [1935], which showed that the heat of oestrogen-treated rats continues undiminished even during the periods of leucocyte smear.

The typical vaginal smear which appeared after prolonged application of gonadotrophic extracts (Plate 1, fig. 2) constitutes an additional feature in which the golden hamster differs from other rodents, and which appears to be characteristic of the species. The fact that this vaginal smear was produced by progesterone and also found in pregnant animals shows that it indicates the luteal phase of the vaginal mucosa. The enlargement of the clitoris, seen in immature hamsters which had prolonged gonadotrophin treatment, also agrees with this view. Steinach & Kun [1931] have observed a similar action of extracts of corpus luteum or of pregnancy urine, on the clitoris of the guinea-pig, and recognized it as the masculinizing effect of the corpus luteum.

The columnar cells found in the smears are, in all probability, analogous to the mucifying cells found in the vaginal wall of mice and rats treated continuously with gonadotrophic extracts, and have been described as denoting 'the second ovarian phase' [Marshall & Wiesner, 1932].

It was not to be expected that, in this respect, substantial differences would be found between chorionic gonadotrophin, with its predominantly luteinizing action, and the anterior pituitary hormone of horses, which is also strongly follicle-stimulating. The experiments were carried out on non-hypophysectomized animals, whose own pituitaries exert an interfering influence [Rowlands, 1938 *a*, *b*; Deanesly, 1939].

G. H. Bell [personal communication] has found that progesterone does not cause any definite luteal phase in the uteri of castrated hamsters 'prepared' with oestrogens. My own results, however, indicated a well-defined luteal phase in the vagina after treatment with progesterone in non-castrated animals, from which the inference may be drawn that the vaginal mucosa is more sensitive in its reaction to progesterone [Klein, 1937] than the endometrium.

In connexion with Bell's interesting results, it should once more be stated that anterior pituitary extract (AP118B) did produce a slight but distinct luteal phase in the uteri of immature hamsters. The difference between Bell's result obtained with synthetic corpus luteum hormone and the described result obtained with AP118B may be explained by the following observation of Klein [1938]. This worker was able to maintain pregnancy in castrated hamsters by simultaneous administration of oestrone and progesterone, which is probably analogous in effect with the twofold action of the anterior pituitary gonadotrophin.

Remarkably enough, large quantities both of chorionic gonadotrophin distributed over several days and of oestrogens, did not result in any enduring disturbance of the oestrous cycle, while anterior pituitary produced continuous anoestrus. However, it is to be noted that the injections of AP118B and of OMAP were stopped in the middle of September and yet, by the middle of October, contrary to expectation, the majority of the hamsters had become anoestrous.

As described, it has been possible to establish two forms of disturbance of the sex function in a relatively large percentage of hamsters: a sterile group characterized by anoestrus, and a second group, in which the animals were sterile in spite of regular oestrous cycles.

The attempts to achieve activation and reactivation in these two groups respectively have so far had this result, that it is in principle possible, by a single injection of moderate amounts of gonadotrophic extract, or of oestrogen, to produce in over

less regular oestrous cycles in the anoestrous group and to induce fertility in both groups. These results also show that in this respect stilboestrol has the same effect as oestradiol.

It was to be expected that in group 3 (sterile animals with regular oestrus) since the follicle-stimulating factor of the hypophysis was not affected, therefore sterility would be influenced by chorionic gonadotrophin alone. It should be noted, however, that the latter extract was effective also in group 4 (anoestrous animals) in which it caused oestrous cycles and pregnancy. It is probable that in this case the animal's own pituitary interfered, causing follicle stimulation as a reaction to the luteinizing effect of the chorionic gonadotrophin. However, it is possible that the positive results would have been more numerous, especially in group 4, if anterior pituitary hormone or a combination of both gonadotrophins [Pincus, 1939; Parkes, 1940] had been used.

Further experiments on this question are in progress, and also as to whether oestradiol is more active than stilboestrol.

Another problem is the possibility of preventing the early death of the hormonally induced litters of the senile females. It is possible that better results might be obtained if the optimum interval between injection and mating [Hammond, 1941] could be determined. As one of the experiments suggests, repetition of the injection at long intervals might also prove helpful.

SUMMARY

Experiments were carried out during their breeding season with a stock of golden hamsters (*Cricetus auratus*) in which a significant percentage of sterility had been recorded.

Oestrus, metoestrus and dioestrus could be distinguished by means of vaginal smears, the results being checked by records of the shape of the ovaries and uterus, the periods of heat, and also by the action of oestrogens and gonadotrophic extracts. In non-castrated animals a typical vaginal smear indicating oestrus was produced both by gonadotrophins and by oestrogens. In castrated individuals, on the other hand, although oestrogens produced an oestrous smear at the beginning of the reaction, this type of smear only lasted for some hours, and then changed to a cornified smear.

Moderate or large doses of gonadotrophins spread over several days caused the production of a typical vaginal smear, which was also found in pregnant hamsters and those treated with progesterone.

A series of injections of anterior pituitary gonadotrophin was followed by inhibition of the oestrous cycle and this inhibition continued indefinitely. Large doses of oestrogens did not produce any lasting inhibition of the cycle but seemed to cause sterility.

Single injections of a moderate dose of stilboestrol, or of chorionic gonadotrophin, induced pregnancy in eleven out of eighteen sterile females. When these females were less than 10 months old, they each produced two or three viable and fertile litters; when above this age, each produced only a single litter, and none of the young lived for more than a few days.

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OESTROGENS AND PRO-OESTROGENS RELATED TO STILBENE AND TRIPHENYLETHYLENE

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It has recently been shown [Emmens, 1941, 1942] that oestrogens may be divided into two classes—those which act directly or with changes that can be effected locally, and for which it is suggested the title oestrogen be retained, and those which apparently need to pass into the general circulation before exhibiting oestrogenic properties, the pro-oestrogens. Oestrogen will, in this communication, refer to the former class.

Two methods are so far available for distinguishing oestrogens from pro-oestrogens. By the first method [Emmens, 1941], the systemic-local ratio (S/L ratio) is determined. This is the ratio between the median effective dose needed in an Allen-Doisy test with spayed mice, when the substance is given subcutaneously in oil, and the median effective dose needed by direct application in 50% water-glycerol to the vaginal canal. For oestrogens the ratio is 50 or more; for pro-oestrogens it approximates to unity. The second method involves the use of spayed mice in which the vagina has been cut into two separate sacs, each with its own opening [Robson & Adler, 1940; Emmens, 1942]. A compound introduced into one of these sacs will, if given in an effective but not excessive dose, stimulate cornification of the sac into which it was introduced, and may or may not cause cornification in the second vaginal sac. If it causes both vaginal sacs to cornify, it is a pro-oestrogen. If it does not cause cornification in the second sac, it is an oestrogen, and has acted locally without entering the circulation in effective amounts.

In the first communication dealing with this subject [Emmens, 1941] it was shown that, of the synthetic compounds examined, diethylstilboestrol, ψ-diethylstilboestrol, and stilboestrols with longer chains than those of two carbon atoms, diethylstilboestrol dimethyl ether, *rac*-hexoestrol and *meso*-hexoestrol, 4:4′-dihydroxy-γ:δ-diphenyl-3:δ-hexadiene, 3:3′:4:4′-tetrahydroxy-γ:δ-diphenyl-*n*-hexane, 1-ethyl-2-(*p*-hydroxyphenyl)-6-hydroxy-1:2:3:4-tetrahydronaphthalene, and triphenylchloroethylene are oestrogens; many other synthetic compounds such as triphenylethylene and α-ethylstilboestrol are pro-oestrogens. With the exception of triphenylchloroethylene and ψ-diethylstilboestrol, the oestrogens are apparently closely related structurally and spatially both to one another and to naturally occurring oestrogens. The anomalous behaviour of triphenylchloroethylene has led to the investigation of compounds related to it and of compounds which bridge the gap between triphenylethylene and and stilboestrols. Other substances related to the series will also be included in this communication.

TECHNIQUE

S/L ratios were determined, as already described [Emmens, 1941] on colonies of spayed albino mice, using thirty or more mice per determination. In some cases, sodium taurocholate was added to the glycerol suspensions, in order to keep them

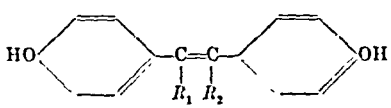
homogeneous. Control tests showed that sodium taurocholate produces no vaginal reactions in concentrations up to 10 mg./ml., and it was used together with the test substances at 1 mg./ml.

RESULTS

Effect of side chains in stilboestrol compounds

A series of stilboestrol compounds is shown in Table 1, and includes many of those originally reported on by Dodds, Golberg, Lawson & Robinson [1939] (stilboestrol = 4 : 4'-dihydroxystilbene). From this table it will be seen that no stilboestrol compound which does not possess two side chains, each as long or longer than those in diethylstilboestrol, is an oestrogen. When the intravaginal doses are compared there is, therefore, a very sharp rise in potency as we pass from methyl-ethyl-stilboestrol, a pro-oestrogen, to diethylstilboestrol. As the side chains lengthen, potency falls, but the S/L ratio remains high. In compounds with only one side chain, however, a low S/L ratio is found, and lengthening of the single chain does not alter the potency very much.

Table 1. *The effect of the side chains on the S/L ratio of derivatives of 4:4'-dihydroxystilbene*



R_1	R_2	Median effective dose in μg. when given: Subcutaneously	Intravaginally	S/L ratio
H	H	2200	> 2000	< 1·0
H	CH_3	700	> 200	< 3·5
CH_3.H	CH_3.H*	650	> 100	< 6·5
CH_3	C_2H_5	0·44	2·2	0·2
C_2H_5	C_2H_5	0·12	0·00937	320
C_2H_5	n-C_3H_7	0·60	0·0035	170
iso-C_3H_7	iso-C_3H_7	4·7	0·015	310
n-C_4H_9	n-C_4H_9	50	0·16	310
H	C_2H_5	90	> 80	< 1·0
H	n-C_3H_7	60	> 60	< 1·0
H	iso-C_3H_7	75	65	1·1

* 4:4'-Dihydroxy-γ:δ-diphenyl-n-butane, included for completeness, as the corresponding stilboestrol compound was not available.

We may conclude, therefore, that in compounds with two aliphatic chains attached to the stilboestrol nucleus, a substance will not be an oestrogen unless it possesses two chains as long as or longer than those in diethylstilboestrol.

Effect of position and number of hydroxyl groups

Table 2 lists a series of comparisons showing the effect of number and position of hydroxyl groups.

In the stilbene series, the high S/L ratio of diethylstilboestrol is lost by the elimination of one hydroxyl group, or by the transposition of the hydroxyls to the 2:2' positions. In both cases there is also great loss of potency. In the benzfluorene series, hydroxyls

at the 3:3′ positions also give rise to great potency loss as compared with the 4:4 compound, and the compound is a pro-oestrogen. Linnell & Sharma [1940] have also shown that 3-hydroxydiethylstilbene, 3:3′- and 3:4′-dihydroxydiethylstilbene are considerably less potent than diethylstilboestrol, and these compounds are probably pro-oestrogens, although they have not been tested.

Table 2. *The effect of hydroxyl groups on the activity and S/L ratio of synthetic oestrogens*

Substance	Median effective dose in μg. when given: Subcutaneously	Intravaginally	S/L ratio
4-Hydroxy-α:β-diethylstilbene	20	30	0·6
4:4′-Dihydroxy-α:β-diethylstilbene	0·12	0·00037	320
2:2′-Dihydroxy-α:β-diethylstilbene	320	165	2·0
4:4′-Dihydroxy-γ:δ-diphenyl-β:δ-hexadiene	0·10	0·00058	170
3:3′-Dihydroxy-γ:δ-diphenyl-β:δ-hexadiene	300	220	1·4
4:4′-Dihydroxy-γ:δ-diphenyl-*n*-hexane (*meso*)	0·16	0·0009	180
3:3′:4:4′-Tetrahydroxy-γ:δ-diphenyl-*n*-hexane	12·5	0·2	63
4:4′-α:β-Tetrahydroxy-diethyl-dibenzyl	30	60	0·5

The effect of additional hydroxyl groups, investigated in the hexane series, depends on their position. A further pair of hydroxyls has been added to hexoestrol (4:4′-dihydroxy-γ:δ-diphenyl-*n*-hexane) in the 3:3′ or α:β positions. Addition in the 3:3′ positions lowers potency, but the compound still has a high S/L ratio. Addition in the α:β positions abolishes the high S/L ratio as well as considerably reducing potency.

It thus appears that a compound of the stilboestrol-hexoestrol group which does not have the 4:4′-dihydroxy configuration is a pro-oestrogen, but that additional hydroxyls may or may not affect the S/L ratio.

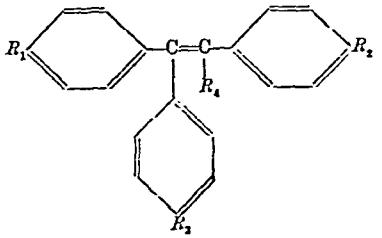
Effect of substituents on the activity of triphenylethylene

Various compounds which may be regarded as linking triphenylethylene and triphenylchloroethylene with diethylstilboestrol are listed in Table 3. The high S/L ratio of triphenylchloroethylene has again been checked, the best estimate now available being a ratio of 86. The substitution of an ethyl group or of hydroxyl groups for chlorine does not give a high-ratio compound, unless both are present in the same molecule.

The compound α-(*p*-hydroxyphenyl)-β-ethyl-stilbene has a high S/L ratio, and α-phenyl-β-ethyl-stilboestrol is very potent intravaginally, with a ratio of 1400. This high ratio, of the same order as that for oestriol (2000), is not affected by the number of subcutaneous injections given, since the percentages of positive responses obtained when 0·5 μg. of the compound was given by two and four injections were 20 and 10% respectively. In this respect it differs from oestriol, which gives a much greater percentage response on increasing the number of injections from two to four [Emmens, 1939].

Since an ethyl group does not effectively replace chlorine in triphenylchloroethylene, the substitution of chlorine for the ethyls of diethylstilboestrol was thought worth trying. The comparison was actually carried out with 4:4′-dimethoxy-α:β-diethylstilbene and 4:4′-dimethoxy-α:β-dichlorostilbene. The former is an oestrogen with an

Table 3. *Compounds linking triphenylchloroethylene and diethylstilboestrol*



R_1	R_2	R_3	R_4	Median effective dose in μg. when given: Subcutaneously	Intravaginally	S/L ratio
H	H	H	Cl	77	0·89	86
H	H	H	H	300	>200	<1·5
H	H	H	C_2H_5	12	4·4	2·7
H	H	OH	H	20	44	0·45
OH	H	OH	H	8·0	20	0·40
OH	OH	H	H	15	10	1·5
H	H	OH	C_2H_5	7·7	0·015	510
OH	OH	H	C_2H_5	0·90	0·00065	1400
Diethylstilboestrol				0·12	0·00037	320

S/L ratio of 400, 8 μg. giving 50% of positive responses by injection, whereas the latter is a pro-oestrogen with an S/L ratio of unity, 340 μg. giving 50% of positive responses by either route. The properties of triphenylchloroethylene are therefore not due to an ability of the chlorine atom to replace an ethyl group, although a phenyl group can do so with little loss of potency (cf. α-phenyl-β-ethyl-stilboestrol). The high S/L ratio of α-(*p*-hydroxyphenyl)-β-ethyl-stilbene demonstrates that in a triphenylic compound, although seemingly not in a diphenylic one, a single *p*-hydroxyl group may confer true oestrogenic activity.

DISCUSSION

It has been supposed that the high potencies of diethylstilboestrol (*trans*), hexoestrol and 4:4'-dihydroxy-γ:δ-diphenyl-β:δ-hexadiene depend on a close structural resemblance to oestradiol [cf. Dodds *et al.* 1939]. It has for some time been a difficulty that *trans*-dihydroxyhexahydrochrysene [Dodds *et al.* 1939] is relatively inactive (M.E.D.[1] = 200 μg.), and that *cis*-diethylstilboestrol (*cis*), which cannot resemble oestradiol in spatial structure (Pl. 1, figs. 1, 2), is highly active. It was also found [Emmens, 1941] that *meso*-hexoestrol is a potent oestrogen (M.E.D. = 0·16 μg.) and that *rac*-hexoestrol is relatively weak (M.E.D. = 8·9 μg.), and the supposition that high potency accompanies a close resemblance to the natural oestrogens and vice versa is difficult to maintain, since models of all forms of hexoestrol can be equally well made to look like oestradiol (Pl. 1, figs. 3, 4). The further discovery that all of the above except dihydroxyhexahydrochrysene have high S/L ratios shows that the activity of the latter is different in kind, as well as in degree, for it is a pro-oestrogen.

The oestrogens so far encountered seem, however, to fall into a single class, in that they all possess or may be said to copy the phenanthrene nucleus. Thus, *cis* diethyl-

[1] Minimal effective dose by subcutaneous injection in mg.

stilboestrol and triphenylchloroethylene, to cite two of the less obvious examples, may take the forms shown in Pl. 1, fig. 2 and in Fig. 5, in which the phenanthrene nucleus is present in skeleton, as in the accepted way of showing diethylstilboestrol (Pl. 1, fig. 1). In addition to having the phenanthrene nucleus, all oestrogens so far examined possess at least one other ring of five or six carbon atoms, or may be arranged spatially so as to copy compounds with such additional rings. True oestrogenic activity then appears to be conferred by a variety of additional factors, outstanding among which are hydroxyl groups and their positions. Another point to be noticed is that flat or nearly flat models may be made of all high ratio compounds, since it appears that the natural oestrogens take as flat a form as possible.

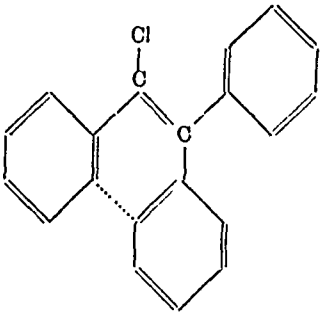


FIG. 5. Triphenylchloroethylene, showing phenanthrene-like structure.

The ability of a single chlorine atom to change the pro-oestrogen triphenylethylene into a more potent, although by no means highly potent, oestrogen is remarkable. The mode of action of triphenylchloroethylene in causing vaginal cornification may be different from that of other oestrogens, but there is no reason to suppose so at the moment. Its prolonged action [cf. Robson, Schönberg & Fahim, 1938], peculiar for a free compound, seems to be related merely to its insolubility in body fluids, and it shows no prolongation of action when given intravaginally. Compared with the majority of oestrogens it is weak, but the lowness of its activity is paralleled by di-*n*-butylstilboestrol, and probably outclassed by members of the stilboestrol series with longer chains. Substances containing chlorine together with hydroxyl groups are difficult to make, but a series containing such groups, based on triphenylchloroethylene, is clearly needed for investigation.

SUMMARY

1. A series of oestrogens and pro-oestrogens related to stilbene and triphenylethylene has been examined. Compounds which exhibit true oestrogenic activity, that they produce cornification of the vagina when injected into it in much smaller doses than are needed subcutaneously, need not necessarily possess a structure which closely resembles that of the natural oestrogens. They appear to have in common a phenanthrene or phenanthrene-like structure, but this similarity may not hold beyond the limited number of compounds examined.

2. Compounds of the stilboestrol series are pro-oestrogens unless they have hydroxyl groups in the 4:4′ positions, and two side chains, each of two or more carbon atoms. Additional hydroxyl groups may or may not affect the classification of a compound, according to their position.

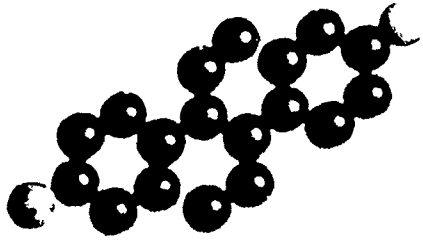


FIG. 1. Model of diethylstilboestrol, showing resemblance which it may have to oestradiol.

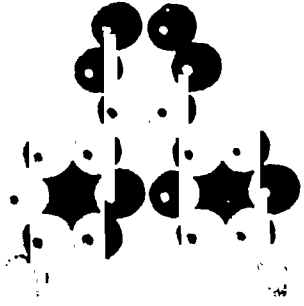


FIG. 2. Model of ψ-diethylstilboestrol, with but little resemblance to oestradiol. The exact disposition of the ethyl groups is conjectural.

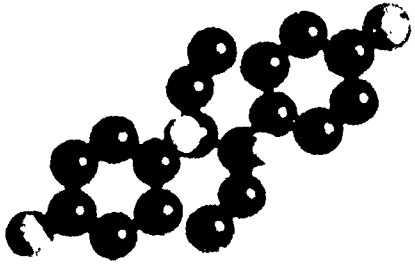


FIG. 3. Model of *meso*-hexoestrol.

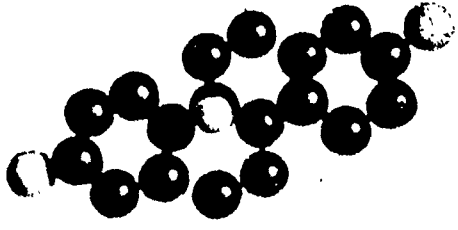


FIG. 4. Model of *l*- or *d*-hexoestrol.

Black = carbon; large fawn = —OH; small fawn = —H.

3. Compounds of the triphenylethylene series exhibiting true oestrogenic activity are described, which possess no hydroxyl groups (triphenylchloroethylene), only one hydroxyl group (α-(p-hydroxyphenyl)-β-ethyl-stilbene), or two hydroxyl groups (α-phenyl-β-ethyl-stilboestrol).

The new compounds mentioned in this communication were generously supplied by Professor E. C. Dodds and Mr W. Lawson. They form part of a series of substances synthesized either at the Courtauld Institute of Biochemistry or at the Dyson Perrins Laboratory, Oxford, under Sir Robert Robinson.

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THE DIFFERENTIATION OF OESTROGENS FROM PRO-OESTROGENS BY THE USE OF SPAYED MICE POSSESSING TWO SEPARATE VAGINAL SACS

By C. W. EMMENS, *From the National Institute for Medical Research, London, N.W. 3*

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Robson & Adler [1940] have demonstrated that oestradiol, diethylstilboestrol and oestriol glucuronide can act locally without absorption into the general circulation. This they did by an ingenious technique, using spayed mice in which the vagina had been separated into two distinct pouches, a blind sac formed from the lower vagina, with the normal opening, and an upper vaginal sac with a suprapubic opening. When the oestrogens were given into the lumen of the upper vagina, this sac alone responded, while the lower vagina was not significantly affected. Even as much as 0·2 μg. of diethylstilboestrol placed in the upper vagina failed to affect the lower vagina.

It has now been shown that oestrogens are of two kinds. The true oestrogens are those which produce vaginal cornification in minute doses when given intravaginally, relative to the amounts needed by subcutaneous injection, whereas other oestrogenic compounds, the pro-oestrogens, must be given into the vagina in approximately as large a dose as the subcutaneous one, to produce equivalent effects [Emmens, 1941, 1942]. The low local activity of the pro-oestrogens is explicable by the hypothesis that they are inert oestrogenically, and must be absorbed into the general circulation, where they give rise to oestrogenic metabolites. It must further be supposed that oestrogens, in contrast to pro-oestrogens, are not distributed throughout the body when given intravaginally in any but very large doses.

The ratio of the median effective dose of a compound when given subcutaneously to that needed intravaginally is the S/L ratio (systemic/local ratio); it is high for the oestrogens, averaging about 300, and approximates unity for the pro-oestrogens.

It so happened that Robson & Adler only used compounds with a high S/L ratio. Had they chanced to test a pro-oestrogen, they should have obtained apparently contradictory results, since their technique is a second method of distinguishing between the two classes of compounds, if the hypothesis outlined above is correct. For it clearly does not matter where a pro-oestrogen is injected into the body (unless into an organ which merely retains or destroys it), if it needs to circulate systemically before it can be metabolized to an oestrogen; and having so circulated, it will affect responsive tissue all over the body.

The hypothesis founded on the S/L ratios was accordingly put to the test by Robson & Adler's method, with the expectation that pro-oestrogens, unlike oestrogens, would cause both vaginal sacs to react although given into only one of them.

TECHNIQUE

A small colony of spayed albino mice, each with two vaginae, was made by Robson & Adler's [1940] method. Only mice with well-formed vaginal sacs of reasonably uniform size were retained for experimental purposes, and used in groups of 5–10 mice

per dose. The upper vaginae held injected fluids better than the lower ones, and were therefore chosen as the site for local application.

Tests were made by a two-injection technique, one injection being given on each of two consecutive days, and smears were taken with a fine metal spatula on the third and fourth days. Substances to be tested intravaginally were dissolved or suspended in 50% water-glycerol and injected in a volume of 0·01 ml. This amount was not always fully retained, but leakage never seemed serious, and did not spread as far as the lower vaginal opening, the only danger entailed. For the purposes of these tests, it does not matter if the actual dose absorbed is rather less than that supposed, as the critical comparisons are made within groups of animals, and not between them, as in the usual tests. In some cases, homogeneous suspension of the active material was greatly facilitated by the addition of small amounts of sodium taurocholate [Emmens, 1942]. Subcutaneous injections were made with nut-oil solutions, the test method being otherwise as before.

Robson & Adler used a scale of responses by which various degrees of vaginal reaction short of full cornification are assessed. In the present tests, as is the practice in these laboratories, vaginal smears were scored only as positive or negative. A positive smear contains no leucocytes, and shows cornified or nucleated epithelial cells, usually the former. However, since some further measure of the degree of stimulation occurring in the lower vaginal sac seemed advisable, when full responses were not elicited therein, a note was made of smears showing submaximal levels of response. We do not find such levels easy to assess, and would hesitate to place much reliance on any interpretation based upon them unless large groups were employed and followed during dioestrus as well as during periods of assumed stimulation.

RESULTS

The results are shown in Table 1. They are in full agreement with the hypothesis tested, and with Robson & Adler's findings. The oestrogens, oestrone, diethylstilboestrol and triphenylchloroethylene, caused no cornification in the lower vagina even when given in relatively large doses; indeed, there was practically no response in the lower vaginae when 200 times the median effective dose of oestrone (0·0003 μg.) was placed in the upper vaginal sac. One mouse alone showed some degree of cornification, with many leucocytes. A dose of 0·2 μg. of oestrone caused cornification in one lower vagina; this dose is approximately 700 times the median effective dose, and would, if injected subcutaneously, have produced a predominance of cornified smears (see below). 0·2 μg. of diethylstilboestrol caused practically no response in the lower vagina, as Robson & Adler found, although the same amount would have produced almost 100% of positive reactions by systemic injection.

All of the pro-oestrogens tested produced cornification of the lower vagina, in addition to causing it in the upper vagina, and when given in minimal doses. In thirty-eight mice which exhibited full cornification in the upper vagina, twenty-three showed cornification of the lower vagina and the remainder showed many cornified cells, while of the twenty-seven mice not responding fully in the upper vagina, three showed cornification of the lower vagina. The responses in the two vaginal sacs were thus correlated, and smears from the upper sac were positive more often than those from the lower sac, although the difference is not statistically significant. When the

Table 1. *Differential action of oestrogens and pro-oestrogens on spayed mice with two vaginal sacs, when given into the upper vagina*

Substance	Dose μg.	No. of mice	No. of positive responses: Upper vagina	No. of positive responses: Lower vagina
Oestrogens				
Oestrone	0·0005	5	3	0
	0·001	5	4	0
	0·004	10	9	0
	0·06	5	5	0
	0·2	5	5	1
Diethylstilboestrol	0·002	5	4	0
	0·02	4	4	0
	0·2	5	5	0
Triphenylchloroethylene	2·0	5	2	0
	5·0	10	8	0
Pro-oestrogens				
α-Phenyl-stilboestrol	15	5	1	1
	30	10	3	2
	100	10	8	8
9:10-Dihydroxy-9:10-di-*n*-propyl-9:10-dihydro-1:2:5:6-dibenzanthracene	40	10	8	3
	100	5	5	5
α-Ethyl-triphenylethylene	10	5	2	0
	30	5	4	2
α-(4-Hydroxy-phenyl)-stilbene	50	10	4	2
α-Methyl-β-ethyl-stilboestrol	2	5	3	3

present criteria of response are applied, the two vaginal sacs do not differ much in sensitivity, as thirty-two mice injected subcutaneously with 0·1 μg. of oestrone showed eighteen positive responses in the lower vagina and sixteen in the upper vagina.

DISCUSSION

These tests have taken us a step forward in confirmation of the hypothesis relating to pro-oestrogens. They demonstrate that the pro-oestrogens are in fact absorbed into the general circulation, and that cornification of the vaginal sac into which they are directly introduced takes place only with the same level of dosage at which such general absorption and action at a distance is demonstrable. The tests fall short of proving that it is metabolic products of pro-oestrogens which cause vaginal reactions; but they add to the strong presumption that such is the case. There is, however, an instance on record in which the conversion appears to have occurred. Stroud [1940] showed that when diphenylhexadiene was injected into female rabbits, a trace of a very highly active phenol, presumed to be 4:4′-dihydroxy-γ:δ-diphenyl-β:δ-hexadiene, was recovered from the urine, in an amount equivalent to 20 r.u. (rat units). The latter is an oestrogen, and the former is practically certainly a pro-oestrogen, although it has not been tested.

The total activity of diphenylhexadiene can be accounted for by Stroud's findings, on the basis of the conversion of a very small part of it to dihydroxydiphenylhexadiene. When he injected rabbits with the latter [Stroud, 1939] he recovered 7·2 % of it in the urine. If the 20 r.u. of oestrogenic activity found in the urine of

rabbits injected with diphenylhexadiene are due to dihydroxydiphenylhexadiene, it may be supposed that this amount also represents only some 7·2 % of the amount of the phenol actually formed in the body, which would be 278 r.u. The rat unit of diphenylhexadiene is 10 mg., and 3 g. of it were injected, or 300 r.u.

In no other case did Stroud find a high S/L ratio compound in the urine, but he found large quantities of pro-oestrogens, in amounts representing 3–25 % of the parent compound injected. It is not improbable that traces of highly active oestrogens may have been present, but masked in the tests by the relatively huge quantities of other phenolic products. Stroud did not test these urines for oestrogenic activity, so no check on the possible presence of such traces is available. The amount of one of the highly potent oestrogens that need have been formed to account for the activity of the parent substances, when these are measurably active, or of the phenolic pro-oestrogens also formed from them, is minute. Thus, stilbene, with 1 r.u. per 25 mg., needs to give only a 0·004 % yield of a highly active metabolite such as diethylstilboestrol to account for its apparent activity. It is not surprising, therefore, that in the presence of other phenols in large amounts, no oestrogens were isolated or suspected to be present, and the data at present available are consistent with their having been formed. It remains to be shown that an oestrogen is in fact regularly formed in small amounts when any pro-oestrogen is injected, and if tests directed to this end prove successful, the complete hypothesis will have been substantiated.

SUMMARY

1. The supposition that pro-oestrogens are compounds which are metabolized in the body and form oestrogens receives support from the fact that, in spayed mice possessing two separate vaginal sacs, they stimulate cornification in both sacs when given in only minimal effective doses into one of them. Oestrogens, on the other hand, can act locally without causing reactions in the second vaginal sac, even when given in many times the minimal effective dose.

2. A case in which the conversion of a pro-oestrogen to an oestrogen has very probably been demonstrated is discussed [cf. Stroud, 1940]. In this instance, the oestrogenic activity of diphenylhexadiene can be accounted for by its conversion in very small amounts to 4:4′-dihydroxy-γ:δ-diphenyl-β:δ-hexadiene.

The synthetic compounds listed in the table were generously provided by Professor E. C. Dodds and Mr W. Lawson. They form part of a series of substances synthesized either at the Courtauld Institute of Biochemistry or at the Dyson Perrins Laboratory, Oxford, under Sir Robert Robinson.

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FURTHER EXPERIMENTS ON LACTATION IN THYROIDECTOMIZED RATS: THE ROLE OF THE PARATHYROIDS

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Despite the unquestioned galactopoiesis evoked in cows by thyroxine or dried thyroid gland [see Folley, 1940, for review] there is little unanimity about the effect of thyroidectomy on lactation [see Folley, 1938]. As regards the rat in particular there is a clear divergence of opinion on this point. Nelson & Tobin [1937] found that their rats weaned normal litters after thyroidectomy performed during pregnancy. Folley [1938], on the other hand, showed that thyroidectomy carried out early in lactation resulted in a prompt reduction in the growth of the pups, only a small proportion of which lived to be weaned and then only because the death of the majority of the young reduced the demands on the depleted milk supply. Nelson [1939] subsequently reaffirmed his original findings, though he now reported a slight retardation of the growth of litters of rats thyroidectomized during the current lactation. Results which may be considered as intermediate between those of Nelson and Folley have been reported by Precheim [1940], who observed subnormal growth of the young of mothers thyroidectomized before mating, but only to the extent of about 12%.

In view of the clear discrepancy between the findings in Nelson's laboratory and in our own, we have, as opportunity from time to time occurred, carried out further experiments on this question over the last three years and now present further evidence to show that in the strain of rat used by us, thyroidectomy, an operation which also involves the removal of, to say the least, a considerable amount of the parathyroid tissue, causes very serious impairment of lactation. Previous attempts at replacement therapy in parturient, thyroidectomized rats failed [Folley, 1938]. Partial success has been achieved in the present series of experiments which appear to give a clue to the cause of the discrepancy between our findings and those of Nelson.

EXPERIMENTAL

Experimental animals

The rats were stock females from the colony of hooded Norway rats maintained for nutritional studies in this Institute by Dr S. K. Kon. Their diet was described previously [Folley, 1938]. As before, lactational performance was judged by the growth and survival rates of litters, unfortunately the only method which is at present available in small animals. To ensure the selection for experiment of only those does in which it was certain that lactation was successfully established, the operation was performed on the sixth post-partum day throughout. The number of

pups was reduced to eight at birth with as nearly equal sex distribution as possible; sometimes it was necessary to use rats with litters of six or seven, but only in two exceptional cases were rats with smaller litters included. Data pertaining to does which died after the operation were not considered.

The groups throughout were smaller than desirable. This was due to the fact that of the limited number of does which from time to time could be made available for this work, a considerable proportion was eliminated by various factors, such as failure to mate, failure to lactate and operative mortality. The second of these factors was at times quite important.

Technique of thyroidectomy

Parathyroid tissue embedded in the thyroid must unavoidably be removed with the latter, but since no attempt was made to find and remove all the existing parathyroid tissue the operation is here referred to simply as thyroidectomy.

Since thyroidectomy in the rat is a more difficult operation to perform satisfactorily than most of the rather casual references to it in the literature would lead one to believe, a detailed description of some points of our technique may be of interest. In the earlier series, as in the previous work [Folley, 1938], the operation was done in a good light with the naked eye, but later a Leitz binocular magnifier giving a magnification of 3·5 × was used and this we now consider essential. For removing the gland we used an iridectomy knife and a pair of eye forceps the points of which were ground fine under the magnifier. With the head of the rat towards the operator, the two lobes of the thyroid were removed in turn, beginning in each case in the middle of the isthmus. Each lobe was carefully dissected from the trachea, working from the caudal end towards the pedicle at its cranial end. It is necessary to dissect the thyroid tissue very carefully from the recurrent laryngeal nerve with which it is closely associated, avoiding injury to the nerve; otherwise respiratory complications and death will eventually ensue.

Owing to the vascularity of the thyroid, haemostasis is important, particularly since profuse bleeding would probably adversely affect lactation. The bleeding which occurs during the stripping of the thyroid from the trachea can be controlled by swabbing with small pads of cotton-wool. Haemorrhage from the inferior thyroid vessels is rarely troublesome and can be controlled by pressure from a cotton-wool swab held in the forceps. When the gland is severed at the pedicle there is usually, even when the pedicle is first crushed in the jaws of curved dental forceps, a gush of blood from the superior thyroid vessels, which, in the initial series of experiments, was controlled by packing with cotton-wool swabs. In later series, before finally excising the gland, a small bulldog clip, the points of which had been ground fine under the magnifier, was applied to the pedicle. If the points were moistened before application there was usually no bleeding when the clip was taken off. By this means it is possible to remove the thyroid with relatively little haemorrhage.

In all series but the last, 'Avertin' given intraperitoneally (1 ml. of 2½% solution per 100 g.) was successfully used as an anaesthetic. In the last series this method had to be abandoned (see below).

In order to obtain evidence for the completeness of the operation, all structures in the thyroid region (trachea, oesophagus, surrounding muscles, etc.) of twenty-three does

were removed and examined in serial sections for fragments of the thyroid and parathyroids. These animals included the experimental series in Table 1 thyroidectomized during April 1941 and also the majority of those in the series *a–g*, in Table 3. In the serial sections varying amounts of regenerating thyroid tissue were found in 86·3 % (25) of cases, while in 13·7 % (4) no thyroid fragments were found in the sections examined. But, as the latter serial sections were in all cases incomplete, we are unable, even in these rats, to state with certainty that thyroidectomy was complete. In general, the regenerating follicles gave distinct evidence of hypertrophy and hyperplasia, but uniformity of structure and functional activity was not, however, exclusively met with. This was especially the case in those animals in which pieces of thyroid were, immediately after removal, implanted under the kidney capsule (see below). In these, thyroid fragments were found, but there was no significant attempt at regeneration, and it is possible that the implants rendered regeneration to some extent unnecessary. The occurrence in our experimental series of so high a percentage of incompletely thyroidectomized animals indicates clearly the difficulty of completely ablating the gland and emphasizes the need for careful histological search for fragments of the thyroid and parathyroids which may be present, for even minute fragments may have definite physiological effects when augmented by the administration of preparations of these glands.

In the same sections, accessory parathyroids were encountered in 17·2 % (5) of the cases examined. There is thus a suggested tendency in our series of rats for the occurrence of accessory parathyroids, but it is clear that no final conclusions are possible at present with regard to the presence of these accessory glands, since the upper portion of the thymus with which the parathyroid anlagen are so closely related was not examined.

Hormone preparations

The parathyroid extract used throughout was Messrs Eli Lilly and Co.'s 'Parathor-mone'. This preparation is standardized in the units of Collip & Clark [1925].

Pure thyroxine-sodium was brought into solution in the usual way and the concentration adjusted so that the daily dose was always contained in 1 ml. of solution.

Lactation in thyroidectomized rats

Data are available for thirteen rats which were thyroidectomized on the sixth day of lactation and received no post-operative treatment. These thirteen rats comprise groups which at various times were controls to groups receiving replacement therapy. The number of pups in each litter and their mean weights on days 6, 16 and 21 are given in Table 1. Data for the sixteenth day are given in this and Table 3 in addition to those for the twenty-first day (weaning) because from about the sixteenth day onward the pups are able to eat the mothers' food and their growth is not due to mothers' milk alone. The weight at 16 days is thus perhaps a better indication of lactational performance than the weaning weight, though the latter is often informative too. The results in Table 1 clearly show that in our rats thyroidectomy performed during lactation immediately caused an almost, but not quite, complete cessation of milk secretion unless one makes the unlikely assumption that the operation causes the secretion of a deleterious substance in the milk or a change in its composition of such a nature as to impair its nutritive value.

Table 1. *Survival and mean weights of litters of rats thyroidectomized on the sixth day of lactation*

No. of rat	Experimental series	No. of pups on day of lactation						Mean wt. of pups (g.) on day of lactation			Condition of doe after operation
		6		16		21		6	16	21	
		♂	♀	♂	♀	♂	♀				
7,230	Nov. 1938	3	5	3	5	3	5	13·5	14·1	14·9	—
7,605	Nov. 1938	5	1	0	0	0	0	14·4	—	—	—
7,234	Nov. 1938	5	3	3	2	0	0	11·6	11·6	—	—
8,016	Mar. 1939	4	4	0	1	0	0	11·0	9·0	—	—
7,939	Mar. 1939	2	4	0	0	0	0	11·7	—	—	Poor. Wheezy
8,581	Sept. 1939	4	4	4	3	3	2	12·3	13·6	12·4	—
8,630	Sept. 1939	5	3	3	1	2	0	13·3	14·8	18·5	—
8,677	Sept. 1939	4	4	1	2	0	0	13·6	12·0	—	Abdomen swollen on seventeenth day
11,561	April 1941	4	4	0	0	0	0	10·3	—	—	—
11,374	April 1941	4	4	0	0	0	0	13·4	—	—	Wheezy
11,244*	April 1941	4	3	4	1	3	1	10·7	13·6	16·3	—
11,335	April 1941	5	3	0	0	0	0	11·1	—	—	—
11,412	April 1941	4	3	1	0	0	0	11·0	—	—	—

Mean weights of litters of a typical group of intact does from this colony are as follows: sixth day, 12 g.; sixteenth day, 32 g.; twenty-first day, 44 g.

* Animal in which accessory parathyroids were found.

Table 2 gives data relating to twelve rats which were re-mated after having undergone thyroidectomy on the sixth day of their second or third successful lactation. Many of them, after the operation, received treatment with thyroxine-sodium or dried thyroid gland combined with low doses of parathyroid extract (1 unit daily) and in three cases prolactin as well. These attempts at replacement therapy were unsuccessful. As before [Folley, 1938], no difficulty was experienced in re-mating thyroidectomized females, but since Nelson & Tobin [1937] and Folley [1938] had noted that such does tend to show symptoms of tetany just before parturition, they were given subcutaneous injections of 1 unit of parathyroid hormone and 0·25 mg. of thyroxine-sodium on four occasions at intervals of 4 days beginning about a week after mating. The results in Table 2 show that in no case did one of these does even begin to rear a litter, though in some cases milk was expressible from the nipples. It will be noted that in six cases parturition was somewhat delayed, confirming the previous observations of Folley [1938] and Preheim [1940].

Experiments with autoplastic thyroid grafts

Folley [1938] had previously reported failure to maintain lactation after thyroidectomy by treatment with thyroxine and parathyroid extract. The maximum dose used of the latter was 1 unit of parathyroid hormone every 4 days, which, since the rats showed no symptoms of tetany after thyroidectomy during lactation and since the tetany exhibited by thyroidectomized rats just before parturition was alleviated by a single injection of 1 unit, was considered adequate to put matters right if the post-operative lactational failure were due to parathyroid deficiency. Other unsuccessful attempts at replacement therapy with a variety of treatments made in the initial stages of this series of experiments have been briefly mentioned above and will not be described further.

Table 2. *Lactational performance of rats thyroidectomized during the previous lactation period*

No. of rat	No. of previous successful lactation periods*	Date mated	Red blood corpuscles observed in vaginal smear†	Date due to litter‡	Date littered	Remarks
7230	3	26. i. 39	9. ii. 39	16. ii. 39	—	Foetuses resorbed
7605	2	23. i. 39	6. ii. 39	13. ii. 39	16. ii. 39	Only two pups seen and these were dead. Doe developed tetany soon after parturition
7234	2	19. i. 39	3. ii. 39	10. ii. 39	13. ii. 39	Two live pups seen; others born dead. All dead on second day. Milk could be expressed from nipples
7517	2	2. ii. 39	16. ii. 39	23. ii. 39	24. ii. 39	Doe showed symptoms of tetany 2 days before parturition. Five pups seen; they were cold and unsuckled. No milk expressible from nipples
7419	2	2. ii. 39	16. ii. 39	23. ii. 39	24. ii. 39	Doe in poor condition 2 days before parturition. Two live and four dead pups seen; former soon died. Milk could be expressed from nipples
7411	1	6. ii. 39	20. ii. 39	27. ii. 39	1. iii. 39	Pups cold and unsuckled
7385	2	29. i. 39	12. ii. 39	19. ii. 39	21. ii. 39	Two live pups seen; both dead on second day
7574	2	5. ii. 39	19. ii. 39	26. ii. 39	1. iii. 39	No pups found but weight loss indicated that doe had littered
7242	2	2. ii. 39	16. ii. 39	23. ii. 39	24. ii. 39	Doe showed symptoms of tetany 2 days before parturition. Four live and one dead pup seen; former soon died. Milk could be expressed from nipples
7378	2	Placed with buck on 20. i. 39	Not seen	—	14. ii. 39	Two live and one dead pup seen; former cold and unsuckled. Milk expressible from nipples
7458	2	22. i. 39	5. ii. 39	12. ii. 39	14. ii. 39	Six live pups, cold and unsuckled. Milk freely expressible from nipples
7335	2	Placed with buck on 19. i. 39	Not seen	—	14. ii. 39	Four dead pups seen. Milk expressible from nipples

* This includes the previous lactation during which the does were thyroidectomized on the sixth day. Lactation was successful up to then.

† The so-called placental sign observed on the fourteenth day of pregnancy.

‡ In this colony does often litter 1 day later than the date considered as 'due'. Pregnancy was therefore prolonged in rats 7605, 7234, 7411, 7385, 7574, 7458.

In view of the uniformly negative results we thought it of interest to attempt replacement therapy with thyroid autotransplants in order to determine whether or not the post-operative decline in lactation was due to some unknown non-endocrine factor.

Six rats were used for this experiment. They were thyroidectomized as usual on the sixth day of lactation, and immediately after removal a piece of the excised thyroid was implanted under the capsule of one of the kidneys. Two of the rats died within a day or so of the operation; the other four recovered and three of them weaned litters, but the fourth lost all her pups on the ninth day of lactation. The usual data relating to the litters of this group are given in Table 3, section *a*, and the mean growth curve of the combined litters of the four rats which survived is plotted in Fig. 1. It will be seen that after an initial period of about 3 days during which the grafts were doubtless becoming established the litters grew steadily. The upward trend of the curve cannot be considered as being accentuated by the death of seven pups between the eighth and ninth days since these pups comprised the entire litter of one rat and the average litter size remained practically unchanged by their death. Owing to shortage of experimental animals at the time this experiment was done, a rat with a litter of only four was included in this group (see Table 3). It might be held that this would tend to give an unduly favourable rate of growth, but against this it may be noted that the average litter size was 6·75 before the litter of seven died and after, 6·66. These results therefore suggest that the lactational failure consistently observed in rats from this colony after thyroidectomy is due to the removal or disturbance of endocrine factors. The three rats which weaned pups were autopsied at the end of the experiment and serial sections cut of the tracheas and of the grafts. The blocks from one of the rats were unfortunately lost; examination of sections of the grafts in the other two showed active thyroid and parathyroid tissue.

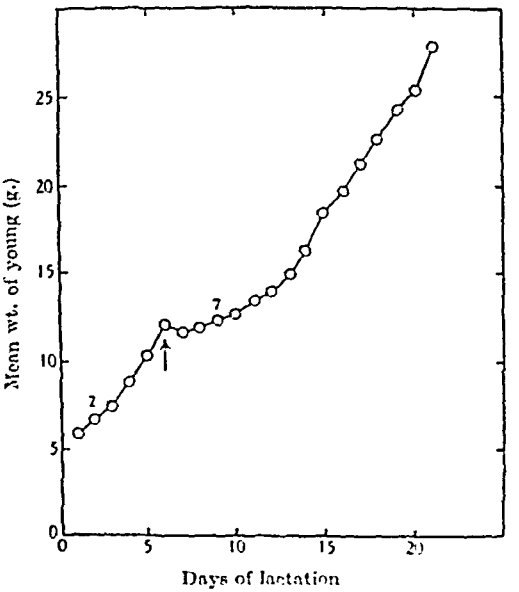


FIG. 1. Mean growth rate of litters of four rats thyroidectomized on the sixth day of lactation and in which immediately after removal, pieces of thyroid were implanted beneath the kidney capsule. ↑ indicates the last observation before thyroidectomy. The numbers above the curve indicate deaths.

Lactation in thyroidectomized rats treated with high doses of parathyroid extract

The intact rat is relatively resistant to the effects of parathyroid extracts [see Thomson & Collip, 1932]. Experiments were therefore carried out in which somewhat higher daily doses of parathyroid extract were administered after thyroidectomy than had been previously tried.

Table 3. *Survival and mean weights of litters of rats thyroidectomized on the sixth day of lactation and thereafter receiving replacement therapy*

No. of rat	Experimental series	No. of pups on day of lactation: 6 ♂	6 ♀	16 ♂	16 ♀	21 ♂	21 ♀	Mean wt. (g.) of pups on day of lactation: 6	16	21	Remarks
(*a*) Rats in which pieces of thyroid tissue were, immediately after removal, implanted under the kidney capsule											
7,999	April 1939	0	7	0	0	0	0	12·1	—	—	—
8,072	April 1939	0	8	0	8	0	8	14·5	22·3	31·3	—
7,876	April 1939	0	8	0	8	0	8	10·9	15·5	21·5	—
8,504	June 1939	1	3	1	3	1	3	10·0	23·3	33·8	—
(*b*) Rats receiving subcutaneous injections of 0·2 mg. of thyroxine-sodium and 5 units of 'Para-thor-mone' daily from the sixth day of lactation, until the pups died or were weaned											
8,686	Sept. 1939	4	4	0	0	0	0	12·5	—	—	—
8,598	Sept. 1939	4	4	0	0	0	0	13·0	—	—	—
8,687	Sept. 1939	4	3	4	3	3	0	12·4	18·9	21·7	—
(*c*) Rats receiving subcutaneous injections of 0·2 mg. of thyroxine-sodium and 10 units of 'Para-thor-mone' daily from the sixth day of lactation until weaning											
8,524	June 1939	0	8	0	6	0	0	14·3	16·7	—	The pups grew until the fourteenth day when the doe suddenly lost 29 g. and the pups began to lose weight
8,398	June 1939	6	0	6	0	6	0	10·8	17·5	19·0	—
8,418	June 1939	8	0	8	0	8	0	12·3	16·8	18·9	—
8,521	June 1939	8	0	8	0	8	0	12·5	17·3	20·0	—

(*d*) Rats receiving subcutaneous injections of 10 units of 'Para-thor-mone' daily from the sixth to the eleventh day of lactation and 20 units daily from the twelfth to the sixteenth day

10,383	Dec. 1940	4	4	1	2	1	1	10·8	21·0	23·0	—
10,373	Dec. 1940	7	0	7	0	7	0	14·3	21·9	29·7	Does showed symptoms of tetany on the eighteenth day; found dead on the twenty-first day
10,214	Dec. 1940	4	4	4	4	3	4	9·1	18·1	18·3	Doe in bad condition from eighteenth day onwards

(*e*) Rats receiving subcutaneous injections of 10 units of 'Para-thor-mone' daily from the sixth to the ninth day of lactation and 20 units daily from the tenth to the sixteenth day in the case of 11,258 and 11,243, and from the tenth to the twentieth day in the case of 11,371 and 11,410

11,258	April 1941	3	5	3	5	3	5	11·9	22·8	26·0	Doe was ill on the nineteenth day and dead on the twenty-first day
11,371	April 1941	4	3	3	3	3	2	12·3	14·2	15·4	—
11,243*	April 1941	3	4	3	4	2	4	10·4	20·3	29·0	—
11,410*	April 1941	4	4	4	4	4	4	10·9	21·3	29·1	—

(*f*) Rats receiving subcutaneous injections of 0·2 mg. of thyroxine-sodium and 10 units of 'Para-thor-mone' daily from the sixth to the ninth day of lactation and 0·2 mg. of thyroxine-sodium and 20 units of 'Para-thor-mone' daily from the tenth to the twentieth day

12,773	Aug. 1941	4	4	3	4	3	3	12·9	18·3	24·8	—
12,979	Aug. 1941	4	3	4	3	4	3	14·7	22·9	29·3	—

(*g*) Rats receiving subcutaneous injections of 10 units of 'Para-thor-mone' daily from the sixth to the ninth day of lactation and 20 units daily from the tenth to the twentieth day

12,867*	Aug. 1941	3	2	3	2	3	2	9·6	25·8	31·4	'Avertin' administered rectally at operation; supplemented by alcohol-ether-chloroform
13,018	Aug. 1941	4	4	4	4	4	4	13·4	21·6	29·4	—

* Animals in which accessory parathyroids were found.

In the first experiment with high daily doses of parathyroid extract (Table 3, section *c*) a trial was made of the effect of 10 units administered daily from the sixth to the twentieth days of lactation in addition to 0·2 mg. of thyroxine-sodium daily. The mean growth curve of the litters of this group is given in Fig. 2. Controls were not available, but since this was an orientating experiment and since untreated thyroidectomized rats had hitherto uniformly failed to rear satisfactory litters, it was considered that lack of controls on this occasion would not invalidate the purpose of the experiment.

The growth curve for the litters of these rats indicated good growth for 3 or 4 days following the operation and institution of the injections, but the curve then flattened out despite the continuance of the treatment. It therefore appeared that the treatment served to maintain lactation for a short time, but that an immunity or resistance to the parathyroid extract quickly developed.

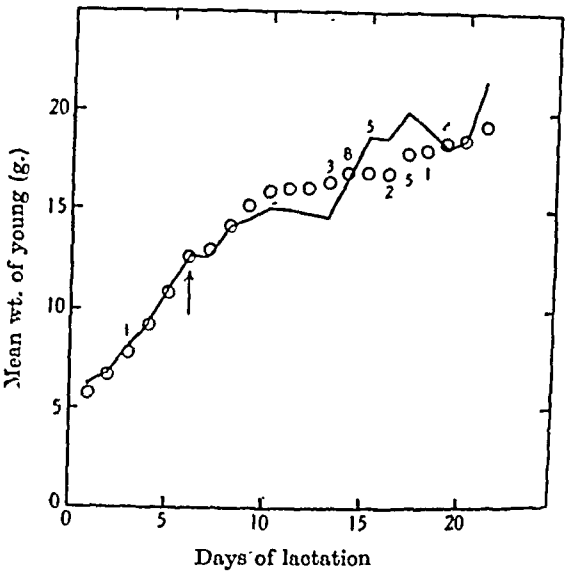


FIG. 2. Mean growth rates of litters of rats thyroidectomized on the sixth day of lactation and thereafter given daily injections of thyroxine-sodium and parathyroid extract. The unbroken curve refers to litters of three thyroidectomized rats receiving subcutaneous injections of 0·2 mg. of thyroxine-sodium and 5 units of 'Para-thor-mone' daily from day 6 until the pups died or were weaned. The circles refer to litters of four thyroidectomized rats receiving subcutaneous injections of 0·2 mg. of thyroxine-sodium and 10 units of 'Para-thor-mone' daily from day 6 until weaning. ↑ indicates the last observation before thyroidectomy. The numbers above the curves refer to the unbroken curve and those below the curves to the circles. These numbers indicate deaths.

A few months later rats were available for a similar experiment, the object of which was to gain some idea of the minimum effective dose of parathyroid extract necessary to maintain lactation for a short time at least following the operation. In this case one group received 5 units of parathyroid extract and 0·2 mg. of thyroxine-sodium daily from the sixth day until the twentieth day or until all the pups were dead, while a second group served as operated, untreated controls (see Table 3 (*b*) for details of the treated group and Table 1, September 1939, series for the controls). The mean growth curve of the litters of the treated rats is given in Fig. 2 for comparison with the curve for the above-mentioned group receiving 10 units of parathyroid extract daily, but in comparing them it must be remembered that the experiments were not done simultaneously. Notwithstanding this proviso, it seems safe to conclude that the lactational performance of the rats receiving 10 units daily was much superior to that of those receiving 5 units. Indeed the latter group was, if anything, inferior in lactational performance to the controls run at the same time. The upward trend of the curve for the 5-unit group at the thirteenth day is due to many deaths among the litters and the latter part of this curve has, in consequence, little significance.

Having established that approximately 10 units of parathyroid hormone daily are

the minimum dose necessary to effect an improvement in lactational performance in thyroidectomized rats and that a resistance to the beneficial effect of this dosage seemed to be established in the course of 3 or 4 days, experiments with even higher doses were planned. Accordingly in the next experiment (see Table 3 (*d*)) 10 units of parathyroid hormone were given daily from the sixth to the eleventh days and 20 units from the twelfth to the sixteenth days. In this experiment no thyroxine-sodium was given.

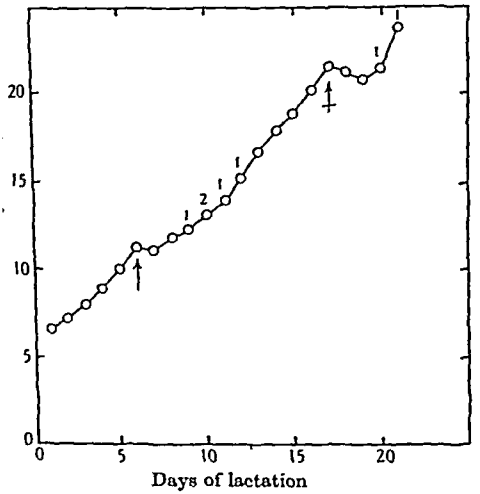


FIG. 3.

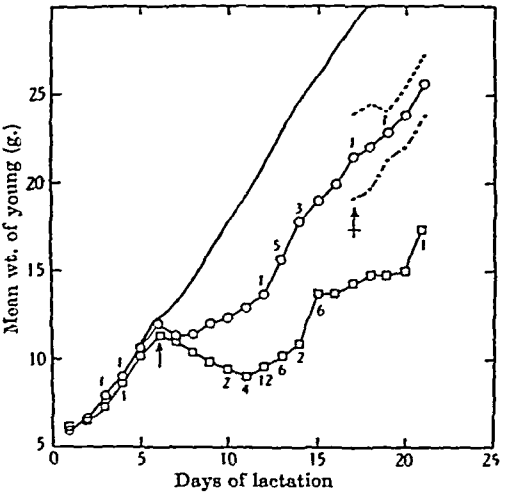


FIG. 4.

FIG. 3. Mean growth rate of litters of three rats thyroidectomized on the sixth day of lactation and thereafter receiving daily subcutaneous injections of 10 units of 'Para-thor-mone' on days 6 to 11 and 20 units on days 12 to 16. † indicates the last observation before thyroidectomy. ± indicates the last observation before injections of parathyroid extract ceased. The numbers above the curve indicate deaths.

FIG. 4. Replacement therapy with parathyroid extract in rats thyroidectomized on the sixth day of lactation. O—O = mean growth curve of the litters of four rats thyroidectomized on the sixth day of lactation and thereafter receiving daily subcutaneous injections of 10 units of 'Para-thor-mone' daily on days 6 to 9. Two of them then received daily injections of 20 units of 'Para-thor-mone' on days 10 to 20; the other two received daily injections of 20 units of 'Para-thor-mone' on days 10 to 16 only. The mean growth curve of the litters of the former pair on days 17 to 21 is represented by ———— and of the latter by - - -. □—□ = curve for the litters of five thyroidectomized, untreated controls. —— represents the growth curve of a group of rats subjected to a control operation on the sixth day of lactation [Folley, 1938]. † indicates the last observation before thyroidectomy. The numbers near the curves indicate deaths.

The growth curve of the litters of these rats (Fig. 3) shows steady and continued growth during the period of the injections, with no indication of a gradual diminution of effect such as was evident in the previous experiment. The rate of growth was however still subnormal. Cessation of the treatment on the sixteenth day was followed promptly by a dramatic decline in the growth rate of the young. Moreover, of the three mothers comprising the group, two were in bad condition on the eighteenth day; one of these showed symptoms of tetany and was dead on the twenty-first day.

Fig. 4 and Table 3 (*e*) give the results of a further experiment in which four thyroidectomized rats were injected with 10 units of parathyroid hormone daily

on days 6 to 9 of lactation; two were then given 20 units on days 10 to 20 and the other two 20 units on days 10 to 16. It was originally intended to begin with a larger group so as to yield two subgroups of satisfactory size; the factors mentioned previously, however, reduced the number available. Data relating to five untreated thyroidectomized controls are given in Table 1 (April 1941 series).

The results again indicate that treatment with high doses of parathyroid hormone results in partial restoration of normal lactation in thyroidectomized rats; it is clear from the figure and the weaning weights in Table 3 that the restoration was, however, far from complete. The figure gives for comparison a curve previously obtained for the litters of a group of rats which underwent a control operation consisting of exposure of the thyroid [Folley, 1938]. The two curves for the subgroups from the seventeenth day onwards indicate a slight decline in growth of the litter of the two rats which were deprived of the extract from the seventeenth day onwards. One of these rats was observed to be ill on the nineteenth day and was dead on the twentieth.

In seeking an explanation for the somewhat better performance of animals 11,243 and 11,410 (Table 3 (*c*)) it is noteworthy that, notwithstanding the incompleteness in the restoration of lactation, accessory parathyroids were found in the thyroid regions of these two animals. Strikingly similar are the data relating to the untreated thyroidectomized controls (Table 1, April 1941 series). In the serial sections of this group, thyroid fragments were present in every case, but it seems significant that the only animal of the group to rear pups to the twenty-first day was found at autopsy to have accessory parathyroids.

A final experiment was run with a view to determining if better replacement therapy could be achieved by post-operative treatment with thyroxine and parathyroid hormone than with the latter alone. Two large groups were started on this experiment, both receiving 10 units of parathyroid hormone on days 6 to 9 and 20 on days 10 to 20. One group in addition received 0·2 mg. of thyroxine-sodium daily throughout the experiment. As the experiment progressed it became evident that both treatments were failing to restore lactation and many more does died soon after the operation than usual. Post-mortem examinations indicated that these deaths were due to peritonitis, for which it seemed possible that the intraperitoneal injections of 'Avertin' might be responsible. This was borne out by the fact that the last four rats of the series (see Table 3 (*f*), (*g*)) in which the 'Avertin' was injected subcutaneously or administered rectally and supplemented by inhalation of an alcohol-ether-chloroform mixture weaned litters. It should be stated that hitherto no ill-effects had been observed following intraperitoneal injection of 'Avertin.' The results of this experiment as far as they go give no indication that treatment with thyroxine in the dosage used, in addition to parathyroid extract, is any improvement on treatment with parathyroid extract alone.

Significant again, however, is the better performance of female 12,867 in this series. This may, at first sight, be ascribed to the smaller size of the litter reared, but since accessory parathyroids were found in the thyroid region of this doe we cannot exclude the possibility that these remnants may have had a definite physiological effect which was enhanced, probably by the augmentation phenomenon, when the parathyroid hormone was given.

DISCUSSION

On the point at issue between ourselves and Nelson, our present results are completely in accord with those reported previously by one of us [Folley, 1938]. In our rats, thyroidectomy performed during lactation causes an almost, but not quite, complete cessation of lactation assuming that the growth and survival rates of litters together form a reliable index of lactational performance. Further, we reaffirm that, though thyroidectomized females can, in the majority of cases, be remated and bear young, satisfactory lactation is rarely established. Moreover, pregnancy is often prolonged and acute tetany usually occurs at the approach of parturition unless preventive measures are taken.

Owing to the close association of thyroid and parathyroids in the rat, it can be assumed that thyroidectomy, as carried out by Nelson and ourselves, will involve the loss of all the parathyroid tissue if it be true, as some maintain, that accessory parathyroid nodules are rarely present in the rat, or of the major portion of it if accessory parathyroids are present. It will be recalled that accessory parathyroids were found in 17·2% of the rats we examined. In either event one would expect that according to the average amount of accessory parathyroid tissue present in the strain of rat used, thyroidectomized rats would suffer from parathyroid deficiency to a corresponding degree. Our evidence for this is satisfactory in the case of thyroidectomized lactating females receiving replacement therapy and, although less complete, points to the same conclusion for thyroidectomized lactating females receiving no exogenous hormone. We have now shown that the failure of lactation after thyroidectomy can be considerably alleviated by continuous administration of fairly high doses of parathyroid extract, which suggests that the post-operative failure of lactation in our rats is indeed connected with parathyroid deficiency.

It is not surprising that there should be such an intimate relation between lactation and parathyroid function when one recalls, on the one hand, the importance of the parathyroid glands as regulators of calcium and phosphorus metabolism and, on the other, the fact that lactation involves a severe strain on the metabolism of these two elements because of the daily withdrawal from the body of large amounts of them in the milk. There is a considerable absorption of calcium from the blood by the active mammary gland [Shaw & Petersen, 1940] and it seems possible that if the mechanism which maintains blood calcium at physiological levels is disturbed, for instance by removal of the parathyroids, the calcium available for the needs of the mammary glands may be insufficient for successful lactation.

Our results are not in agreement with the contention of Dragstedt [1927] that lactation is a more effective stimulus than pregnancy in converting a latent parathyroid deficiency into acute tetany. In rats of the strain used by us, it would seem that the opposite is true. It is, at first sight, difficult to see why in our thyroidectomized rats the tetany evoked by the approach of parturition can be relieved by doses of the order of 1 unit of parathyroid hormone, while the post-operative lactational failure can only be put right, and then only partially, by 10 or 20 times that amount repeatedly administered. The former emergency is, however, evidently temporary, in this respect resembling milk fever in the bovine, a syndrome analogous to tetany in many of its symptoms and accompanied by a fall in serum calcium, while the latter

must be prolonged. In pregnant females, initiation of lactation with the approach of parturition, involving as it does increased demands on the stored calcium, requires that the serum calcium must be rapidly replenished from the calcium reserves. As yet, however, calcium is not being lost in the milk through suckling, and it may be presumed that a dosage of the order of 1 unit of parathyroid hormone suffices to maintain the health of the does. In lactating females, on the other hand, where the strain on the metabolism of this element is greater, because of its daily withdrawal in the milk, the health-maintaining dose for pregnant females will no longer suffice.

To summarize, in our rats as in those used by Nelson, the operation appears to produce a condition of latent parathyroid deficiency which is exacerbated to acute tetany under the stimulus of approaching parturition, but in neither strain of rats does lactation call forth symptoms of tetany. In our experiments, however, but not in Nelson's, the operation sets up conditions which preclude normal lactation.

In the light of the new information, the difference between our results and those of Nelson may reasonably be explained as due to one of the following possibilities. First, the use of rats of different strains which might, on the average, differ in the amount of accessory parathyroid tissue normally present. Secondly, it is possible that Nelson used older rats than we did. According to Dragstedt [1927] the older the animal the more resistant it is to the removal of the parathyroid glands. Thirdly, the difference may be due to some factor connected with diet, since it has been shown by Patras, Galapeaux & Templeton [1938] that the mortality and incidence of tetany in rats after thyro-parathyroidectomy is dependent both on pre- and post-operative dietary management.

Another interesting point which calls for comment is that rats in which lactation had been maintained after thyroidectomy by continuous treatment with parathyroid extract, usually lost condition and often exhibited tetany if the treatment was withdrawn before weaning. If it were true that our rats have sufficient accessory parathyroid tissue to keep them free from tetany after thyroidectomy, these results would be explicable on the assumption that this tissue had undergone hypoplasia and become inactive during the period of exogenous administration. Many similar instances of this are known. On the other hand, where no accessory tissue is present, the tetany might be ascribed to the abrupt withdrawal of the exogenous hormone, resulting in a sudden upset in a calcium and phosphorus metabolism temporarily stabilized during replacement therapy.

It must be remembered that we have not yet succeeded in completely restoring lactation after thyroidectomy and it is probable that the resulting thyroid deficiency must also be corrected before lactation can be completely maintained at the pre-operative level. The evidence offered by our experiments on this point, which, it must be admitted, is rather scanty, is to the effect that thyroxine in addition to parathyroid extract effects no improvement over that due to the latter alone. However, in the absence of reliable information as to the daily dose of thyroxine necessary to restore to normal the metabolic rate of thyroidectomized rats, a decision as to how much, if any, of the lactational failure after thyroidectomy is due to loss of the thyroid is a matter for future experiment.

SUMMARY

1. Thyroidectomy during lactation in our rats immediately causes an almost complete cessation of litter growth and presumably, therefore, of lactation. Thyroidectomized rats will mate and deliver young but seldom begin to rear litters; pregnancy is often prolonged. These results confirm previous findings of one of us (S. J. F.) and differ from those of Nelson.

2. Autoplastic thyroid grafts, containing parathyroid tissue, made immediately after thyroidectomy partially maintained lactation.

3. Lactation was also partially maintained in thyroidectomized rats by injections of high daily doses (10 and 20 Collip & Clark units) of parathyroid extract.

4. Rats thyroidectomized during lactation do not exhibit symptoms of tetany.

5. It is concluded that the failure of lactation after thyroidectomy is due, at least partly, to parathyroid deficiency.

6. In the light of (5) possible explanations for the divergence between our results and those of Nelson are discussed.

We are indebted to the Agricultural Research Council for a research grant to one of us (H. M. S. W.). It is a pleasure to express our thanks to Dr S. K. Kon for placing the facilities of his rat colony at our disposal, and to his staff for care of the rats. Part of the 'Para-thor-mone' was kindly supplied by Messrs Eli Lilly and Co., Ltd.

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ADDENDUM. Since this paper was submitted for publication we have seen a paper by Karnofsky [1942] which describes results substantially in agreement with those reported by Folley [1938]. As our present results indicate, we are able to confirm Karnofsky's opinion that complete thyroidectomy is extremely difficult to achieve in the rat.

EFFECTS OF OESTROGEN (STILBOESTROL) ON THE SPERM PRODUCTION OF ADULT RAMS

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The production of spermatozoa depends on environmental factors, such as climate, season, nutrition, etc., and on internal factors, such as general health and endocrine secretions. These latter secretions play a very important part in the production of germ cells, and a study of their influence on sperm production should be of great value in both human and veterinary medicine. The present experiment was planned to investigate the influence of oestrogens on the sperm production of normal adult rams. The synthetic oestrogen diethylstilboestrol was used throughout.

MATERIAL AND METHODS

Two Suffolk rams (nos. 84 and 86), 3 years old, were used in this experiment. They were kept in a field at the Animal Research Station, Huntingdon Road, Cambridge. The general management was the same as on any farm in south-east England. They were in fairly good condition, and their body weight was almost constant throughout this study. They have been used for the collection of sperm for about two years in connexion with a previous study.

One ewe, which was not necessarily on heat and which was pregnant during the later part of this experiment, was used in the service crate for the ram to mount. An assistant led the ram to the crate and recorded the 'reaction time', i.e. the interval between ascending the platform of the crate and ejaculation. This reaction time was recorded in order to measure the sex drive. On mounting the ewe, the ram usually ejaculated within 2 sec. or as soon as his penis was directed into the artificial vagina by the experimenter.

Semen was collected twice every morning from each animal. The interval between collections was about 5–7 min. If a ram did not jump within 10 min. of leading him to the crate, he was considered as having failed in the trial. He was allowed to have a 10 min. rest and then led to the crate for another collection. No further trial was made if he failed at this second chance.

The temperature of the artificial vagina [Walton, 1938] was adjusted to 40° C., and the tightness of the rubber tube was ascertained by inserting a thumb. By experience, we noticed that temperature and pressure are very important factors in inducing a good ejaculation. Thus control of temperature and pressure is essential at every collection in order to standardize the technique. A vacuum cup is attached to one end of the artificial vagina to receive the semen, and the semen is manipulated in a warm chamber at 25–30° C. in order to prevent temperature shock [Chang & Walton, 1940].

The volume of each ejaculate was measured and the density, that is, the number of spermatozoa per ml. of semen, was estimated from a mixture of the first and second ejaculates. A 1 to 10,000 dilution of sperm was made with normal saline, and the haemocytometer used to count the number of spermatozoa.

The total number of spermatozoa in 48 squares of the haemocytometer was counted and also the proportion of abnormal spermatozoa and those with a protoplasmic droplet. The morphology of abnormal spermatozoa has been described by various authors [McKenzie & Berliner, 1937]. For the sake of simplicity and definition only those spermatozoa without tail, with deformed head or with small head, were classed as abnormal. By spermatozoa with droplet are meant those with the protoplasmic remnant still attached to the middle piece of the tail. The position of the droplet was not taken into account.

The motility of the spermatozoa was tested by dropping 0·1 ml. of semen into 4·9 ml. of glucose-phosphate solution which contains Na_2HPO_4 (anhyd.) 0·824 g., KH_2PO_4 0·08 g., glucose 3·20 g. and glass-distilled water 100 ml. The suspension was left in a warm chamber at 37° C. and examined under the microscope at this temperature. Occasionally the respiration of the spermatozoa was measured in the Dixon-Barcroft respirometer [Dixon, 1934], according to the technique of Chang & Walton [1940]. The keeping quality of the sperm was tested by slow cooling (at 30–25° C. for ½ hr., at 15° C. for 1 hr., at 5° C. for 2 hr.), and finally storing for 10 days at 1° C. [Chang & Walton, 1940]. The fertility of the spermatozoa was tested by actual artificial insemination [Walton, 1938].

Experiments with ram 86 started on 2 November 1941. The semen was collected daily for 12 days before treatment in order to reveal the normal production of spermatozoa. Then ten tablets of diethylstilboestrol (total weight 0·235 g.) were implanted into the loose connective tissue of the inguinal region of the right hindleg. Daily collection of sperm was continued without interruption. The ram reacted quite well and showed no effect of the operation. After 19 days another thirty tablets (total weight 1·477 g.) were implanted into the connective tissue of the inguinal region of the left leg. Ram 84, after 11 control days, received on 22 November 1941 forty tablets (total weight 1·973 g.) implanted in the inguinal region of right leg. Both rams continued to give daily collections and sex drive was maintained. After 31 days of daily collection, 15 mg. of diethylstilboestrol dipropionate dissolved in nut oil was injected subcutaneously into each of the rams. Collection of sperm was made for another 17 days and the tablets were removed and weighed. Unfortunately, ram 84 did not recover well from this removal and had to be rested for 7 days. Semen was collected as usual for 23 days after the removal of the tablets. The collection was stopped on 11 February 1942.

RESULTS

The data on the sperm production before treatment, during treatment and after removal of the tablets are presented in Figs. 1 and 2 for rams 84 and 86 respectively. In order to eliminate minor daily fluctuations, the results are grouped into 5-day periods. The total number of sperms is the sum of all sperms ejaculated in each 5-day period. The volume, the density, the reaction time and the percentage of abnormal sperms are the averages of the ten ejaculations in the same period.

The total number of sperms collected in the first 5 days is more than that in the second 5 days before treatment. This is due to the accumulation of sperms after a few days sexual rest before the experiment began. Sperm production reaches a normal level in the second and third 5-day periods.

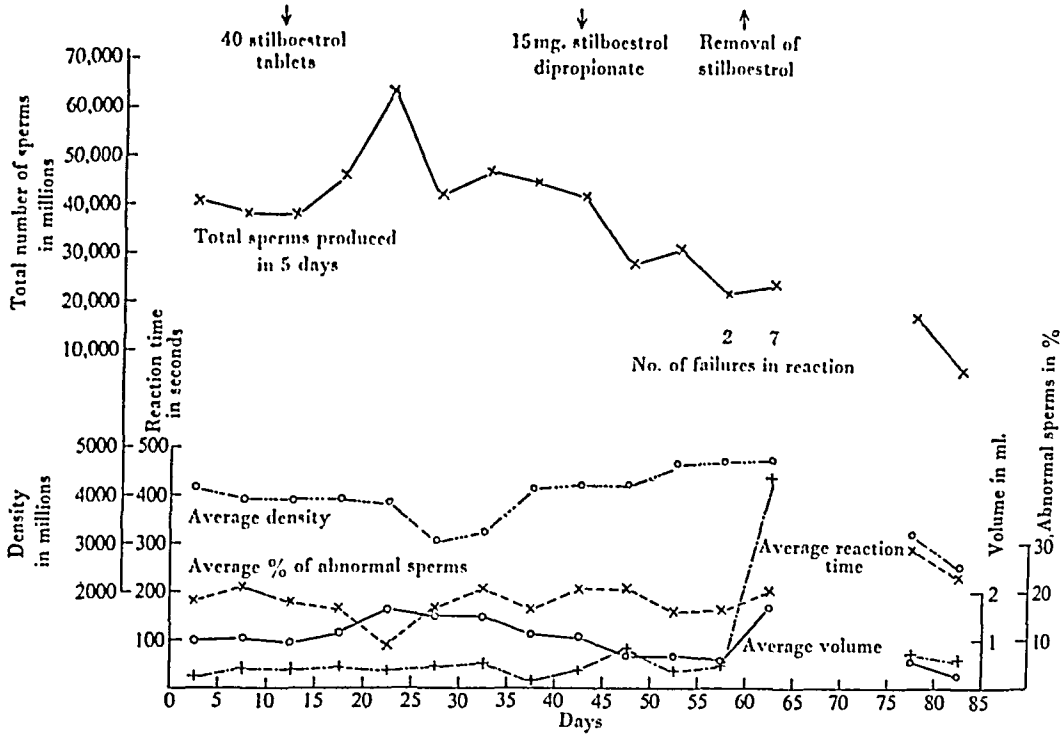


FIG. 1. Effects of stilboestrol on sperm production in Ram 84.

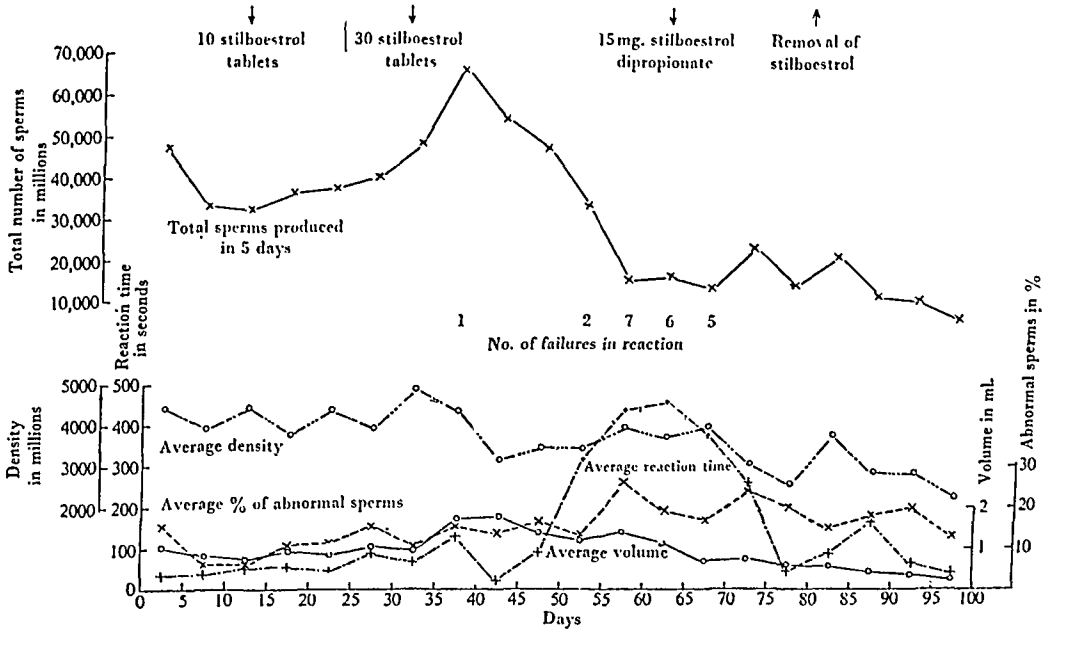


FIG. 2. Effects of stilboestrol on sperm production in Ram 86.

After implantation of stilboestrol there is a definite increase in sperm production, especially when the dosage is high. Curves presented in Fig. 3 show the actual number of sperms produced per day after implantation of stilboestrol and the effect is obvious. The increase, however, does not appear immediately after the implantation; in the case of ram 86 the effect occurs 7 days after each of two consecutive implantations. In the case of ram 84, the increase occurs 9 days after implantation. Increased sperm production, however, only lasts for a short time, on the average about 5 days: 5 days in the case of ram 86 when ten tablets were implanted; 3 or 7 days in the same ram when another thirty tablets were implanted; 4 or 5 days in the case of ram 84 when forty tablets were used.

The effect of injection of stilboestrol dipropionate is not very conspicuous and may not be significant. The sperm production and sex drive of ram 86 were declining at the time of the injection. He even refused to jump many times before injection and for

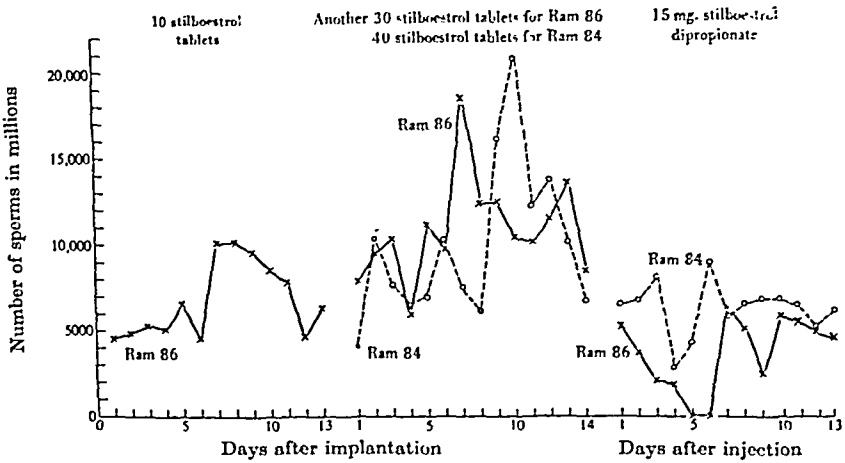


Fig. 3. Daily sperm production during stilboestrol treatment.

6 days after injection. But he mounted for service vigorously and ejaculated on the seventh day, and this may have been due to the effect of the injection. Sperm production was maintained for many days. Although the sperm production of ram 84 was also declining in this period sex drive remained normal. Sperm production was not very conclusively increased after injection. This may be because the dosage was not high enough in the case of the injection. There appears to be a definite relation between dosage and effect in the case of the tablets. The curves in Fig. 3 show the greater effects with the higher dosages.

After the removal of the tablets, the total sperm produced in 5 days, the average volume and the average density decreased. The quality of the sperms was also low (see below). As the sperm production of the ram is subject to seasonal variation [McKenzie & Berliner, 1937; Chang, 1941] it is difficult to say whether this was due to the removal of stilboestrol or due to seasonal changes. Curves in Fig. 4 represent the data collected in February 1941 and February 1942 after the tablets had been removed. In 1941 the daily collections were made after about 7–10 days' sexual rest for the two rams, so the total number of sperms is higher in the first and second days.

In 1942 only ram 84 got a rest of 7 days for operative recovery, so the number of sperms in the first and second days is high; ram 86, however, had semen collected daily after the removal of the tablets and therefore did not have a high count in the first two days.

By comparison of the curves it seems that the sperm production of the rams after removal of stilboestrol is lower than that of a normal ram at this season of the year. However, collection from these animals in 1941 was not so systematic as in 1942; therefore I cannot definitely claim that the removal of stilboestrol was the cause of the decline of sperm production.

The density and the volume of the samples of semen increase during treatment (cf. Figs. 1, 2), the increase in the volume of semen being the more marked. The ram usually ejaculates about 1 ml. of semen if regular collections are made with an

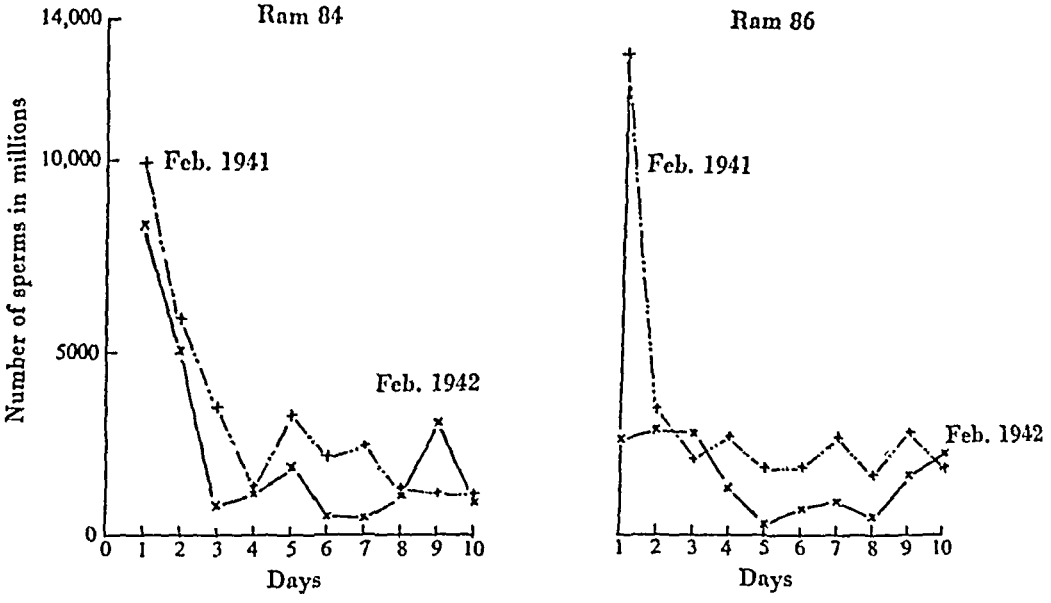


Fig. 4. Daily sperm production after removal of stilboestrol tablets.

nterval of not more than 3–4 days. After a longer period of sexual rest the amount nay rise to about 1·5 ml. In the rams subjected to stilboestrol treatment the volume ·ose in some instances to 2 or even 2·5 ml. This increase in volume certainly indicates ın effect of the treatment on the accessory sex glands.

Abnormal sperms usually occur in high percentage after a lengthy sexual repose ınd also in the non-breeding season. The percentage of abnormal sperms before and luring treatment does not show much change. In the case of ram 86 the percentage s a little higher than normal in the later part of this experiment, but this may be ıscribed to seasonal variation.

The percentage of 'droplet' sperms is not shown in the graphs, but it changes ·ery little during treatment. In the case of ram 86 the average percentage of droplet perms per 5-day collection during the first 25 days ranged from 8·4 to 13·5, in the ıext 30 days from 1·1 to 6·6, while afterwards it kept at a level of about 1 %. After emoval of the tablets the percentage of 'droplet' sperms increased to 4. In most ases the droplet was on the neck. In ram 84 the percentage in the first 25 days

was 1·3–3·3 per 5-day collection. In the next 30 days, the percentage was 1·3–3, and later 1·1–3·1. After removal of the stilboestrol, the droplet was on the neck of spermatozoa in this ram also. Thus it is not clear whether or not the female hormone has any influence on the percentage of droplet sperm. If there is any effect there may be a slight decrease after implantation.

The reaction time is a measure of the sexual drive of the animal. The reaction time of the first collection of each day was shorter than that of the second collection. Table 1 shows the difference, which probably represents the fall in sex drive after gratification in the first ejaculation.

Table 1

		Reaction time in seconds				Reaction time in seconds	
		1st collection	2nd collection			1st collection	2nd collection
Ram 84	Average	20	85	Ram 86	Average	47	110
	Range	7–61	20–133		Range	9–264	25–244

Concerning the reaction time before and during treatment, not much difference was observed in the case of ram 84. In the case of ram 86, 20 days after the second implantation the reaction time was very high which indicates a low degree of sexual drive. This ram only reacted 3–8 times out of the ten trials in every 5 days during this period. Since stilboestrol tablets still remained in his body when he reacted quite well later, the failure to react may be due to some general body condition other than the effects of stilboestrol.

The motility of the sperms suspended in glucose-phosphate and examined at 37° C. under the microscope showed no difference before and during treatment. In most cases, about four-fifths of the sperms showed progressive motility, which was maintained for about 6–8 hr. at 37° C. Motility ceased after 24 hr. at this temperature. This performance does not differ from that of normal rams.

One difference was, however, observed in that the sperms from treated animals agglutinated immediately or within 1 hr., when the suspension was kept at 37° C. Sperms collected from normal animals sometimes agglutinate, but only after 6 hr. under the same conditions. A peculiarity of this agglutination during stilboestrol treatment is that the agglutinations may contain 50–100 or more sperms in each group, heads sticking together and tails waving, after 2–3 hr. at 37° C. Such heavy agglutination has not been observed before.

This heavy agglutination is not due to *p*H changes as is shown by the following facts. First, the sperms of ram 86 have a high tendency to form heavy agglutinations while those of ram 84 have a low tendency, although the *p*H of the sperm suspensions of both rams is the same (about 7·6). Secondly, when a reagent[1] was added to the suspension to inhibit the *p*H change of the sperm suspension, the sperms still agglutinated although the suspensions were of different *p*H value. Thirdly, the sperm collected from an untreated ram (no. 128) showed no agglutination, although the *p*H change of the sperm suspension was the same as that of the experimental animals.

[1] The action of certain inhibitors of sperm activity is being studied and results will be published subsequently.

The motility of the sperm after removal of stilboestrol was less than what it was before. In most cases only one-fifth of the sperms showed progressive motion. The agglutination phenomenon also disappeared after removal of stilboestrol.

The results on the respiration of the sperms are represented in Table 2. These indicate that there is no difference before and during treatment. They show, however, that the respiration of sperms after removal of the stilboestrol was rather poor. The respiration tests therefore confirm the results obtained on motility.

Table 2. *Respiration of ram spermatozoa before, during and after stilboestrol treatment (oxygen in µl., consumed per million spermatozoa)*

Condition of animals	Ram	Average oxygen uptake		
		1st hr.	2nd hr.	Total
Before treatment (two measurements)	84	0·252	0·240	0·492
	86	0·298	0·244	0·542
During treatment (two measurements)	84	0·251	0·250	0·501
	86	0·330	0·238	0·569
After removal of stilboestrol (two measurements)	84	0·171	0·128	0·298
	86	0·168	0·109	0·277
In normal condition, February 1941 (ten measurements)	84	0·244	0·205	0·449
	86	0·279	0·253	0·530

Table 3 shows the keeping quality of sperm before and during treatment of the rams. By keeping quality, we mean the ability to remain motile at a low temperature for a long time. It is known from experience, that the keeping quality of sperm varies not only in different species and in different animals but also in different ejaculates. There was little difference between the keeping qualities of the sperm before or during treatment, but, if significant, the data may show that after treatment the keeping quality is reduced.

Table 3. *Keeping qualities of sperm before and during stilboestrol treatment of rams (pure sperm stored at 1° C. for 10 days)*

Ram	Before treatment			During treatment		
	No. of sample	*p*H after storage	Motility, diluted with G.P.S.*	No. of sample	*p*H after storage	Motility, diluted with G.P.S.
84	1	5	2/5–3/5 sluggish	1	5	4/5 active
	2	5	A few motile	2	5	None
	3	5	2/5 sluggish to active	3	3	None
86	1	5	1/5–2/5 sluggish	1	5	None
	2	5·5	2/5 active	2	4·5	None
	3	5	2/5 active	3	3	3/5 motile

* Glucose-phosphate solution.

Sperms collected from the ram during the period of stilboestrol treatment were fertile. Five ewes inseminated with sperm collected from ram 84 and ram 86 during treatment were impregnated and gave birth to 8 normal lambs; 5 males, 3 females.

The ten stilboestrol tablets implanted into ram 86 were totally absorbed 67 days afterwards when it was intended to remove them. It is impossible to calculate the absorption rate because we do not know the actual time of total absorption. 204 mg.

of the other thirty tablets were absorbed after 48 days. The absorption rate was therefore 4·25 mg./day. In ram 84, 240 mg. of stilboestrol were absorbed after 48 days of implantation. The absorption rate was 5 mg./day.

DISCUSSION

The results of this experiment are not in accordance with my original expectation. Golding & Ramirez [1928] demonstrated that the injection of oestrogen from ovaries and placenta into immature rats would inhibit the growth of testes and prevent descent into the scrotum. Ringoen [1938] has also reported the retrogression of the gonads of sparrows following injection of oestrogen. The implantation of stilboestrol into ram lambs by Hammond (experiment still in progress) also caused inhibition of growth of the testicles. Thus I assumed that sperm production would be stopped by implantation of stilboestrol but might return after the removal of the hormone. The results, however, show a definite increase in sperm production and no decrease in the sperm quality. We know from recent literature that there are definite periods of competence when induction occurs in developmental processes; therefore it seems that the influence of the female hormone on the male organs may occur only at certain stages of development.

It is a well-known fact that high temperature applied to the testicles adversely affects spermatogenesis. The time interval between administration and effect is, however, rather long. Thus abnormal sperms appear after 8·8 days in the case of rams [McKenzie & Phillips, 1934]; fertility decreases after 44 days in the case of the guinea-pig [Young, 1929]; and low sperm counts occur after about 21 days in man [McLeod & Hotchkiss, 1941]. I thought at the beginning of this experiment that the effect of stilboestrol, if it caused damage to spermatogenesis, would come after a long period of treatment. The experiment has been carried on 71–90 days after the administration of stilboestrol, but there is still no definite sign of any ill effect. The experiment cannot, however, be continued longer, as at this time of the year decrease in testicular activity is to be expected owing to the onset of the non-breeding season. However, sperms showed normal quantity and quality 20 days after the experiment was stopped.

Recent literature reveals that there is similarity in the chemical structure of the sex hormones. Oestrogenic and androgenic substances are produced in both males and females. This is responsible for a certain degree of overlapping in reaction. Testosterone, the hormone of the testis, produces not only development of male secondary sex organs, but in large doses has an action on the uterus similar to that of progesterone [Klein & Parkes, 1937]. Testosterone also has an effect similar to that of oestradiol on the vagina of the immature mouse [Robson, 1940]. That the oestrogens may stimulate a certain type of tissue development in the seminal vesicle, prostate, or peri-urethral tissue of the male has been confirmed many times [Gustavson, 1939]. Our results reveal that stilboestrol has no ill effects on the adult male organs and that it temporarily increases the sperm production of an adult ram. Although the effects of synthetic oestrogens may not be exactly similar to those of natural oestrogens which belong to the steroid group, the similarity of effect as well as the close structural resemblance of diethyl stilboestrol to oestrone or oestradiol are well established. Thus the results of the present experiment may be applicable in general to other oestrogens.

It is claimed that the action of oestrogens is that of a pacemaker in the action of male hormone in the castrated rat and that oestrogen and androgen act synergistically on the epithelial and smooth muscle elements of the seminal vesicles [Gustavson, 1939]. Although the administration of androgen maintains spermatogenesis in hypophysectomized animals [Walsh, Cuyler & McCullagh, 1934; Nelson, 1936], in general, androgens are considered to stimulate only the accessory secretions and sex drive; their relation to spermatogenesis in normal conditions is not altogether clear. The present experiment shows that the volume of semen and the number of spermatozoa increase after treatment with stilboestrol, and indicates that stilboestrol affects both accessory secretion and spermatogenesis. But to what extent it stimulates accessory secretion and spermatogenesis directly and what is the relationship between the stimulation of accessory glands and that of spermatogenesis is still not yet clear. It is possible that the implantation of stilboestrol stimulates the pituitary to produce more gonadotropic hormone which therefore causes the increase of sperm production and accessory secretion.

That the administration of oestrogens prevents the castration changes in the hypophysis has been well demonstrated [Nelson, 1934; Wolfe, 1936]. In a review of the recent literature on the effect of the administration of oestrogens on the hypophysis, Severinghaus [1939] summarized the position by stating that 'the structural response of anterior lobe tissue to oestrogen administration, judged by the best available cytological criteria, indicates an activation of production and release of secretion'. Thus the results of the present experiment could be interpreted by saying that the stilboestrol stimulates the activity of the pituitary. The increased activity of the pituitary gland may either increase the production or release the secretory mechanism of gonadotropic hormones which stimulate spermatogenesis and the accessory secretions. If this interpretation is right it seems more probable that the effect of stilboestrol is due to the release of hormone present in the pituitary rather than to the maintenance of a high level of hormone production, because the increase of sperm production only lasts for 5 days, although stilboestrol is still being absorbed into the body.

Actually, the release of gonadotropic hormone from the pituitary may have an immediate effect on spermatogenesis, but this would not appear immediately in the ejaculate. The transportation of spermatozoa from testicle to the tail of the epididymis is rather slow; 8·8 days as determined by McKenzie & Phillips [1934], 5–6 days according to Gunn [1936]. The time interval between administration of stilboestrol and the increase of sperm production which occurs 7–9 days later may be due to the time required for the transportation of spermatozoa rather than to a long latent period between administration and effect on the pituitary or gonad.

The definitely low quality of the spermatozoa and the relatively low sperm production after the removal of stilboestrol, and the fact that the protoplasmic droplet is in most cases on the neck, which is claimed by some authors as an indication of immaturity of spermatozoa, could be ascribed to the seasonal effect on sperm production in part, but also to a greater extent to the effect of removing the stilboestrol. It is most probable that the sudden removal of a constant stilboestrol stimulation reverses the release of secretion for a certain period. During this period the normal amount of gonadotropic hormone would not be secreted, hence the low sperm production and low quality of spermatozoa.

That the inunction of stilboestrol causes udder development and copious secretion of normal milk in virgin goats and heifers has been demonstrated by Folley and his collaborators [Folley, Scott Watson & Bottomley, 1940, 1941]. They claim that prolactin treatment before administration of oestrogen is not necessary, although this has been postulated by other authors. Their results, like ours, point to the same chain of reactions between the pituitary and sex hormones, though the mechanism of these reactions is still not clear.

The methods and procedure in collecting sperm, which have been described above, were planned to reveal the absolute production of spermatozoa. The method is not, however, an obligatory process like milking a cow. Success in collection depends partly on the sex drive of the animal. If the sex drive is low the ram will refuse to mount, and the sperm production during this period could not be revealed even though it might be on a high level. Moreover, the total number of sperms produced in a given period increases as the number of collections increases [Chang, 1941], while at the same time the sex drive may diminish with increased gratification. It is very difficult to separate these two factors, sex drive and spermatogenesis, especially as they are also controlled by different gonadotropic hormones which may however be related to one another. We hoped that by making two collections each day we would obtain sufficient elimination of sperms to reflect the level of production without at the same time reducing the sex drive by increased gratification. In the future an obligatory method of collection might be used to measure the real production of sperms without introducing the handicap of low sex drive.

The phenomenon of agglutination of sperms, which is still under investigation, may be due to the direct effect of stilboestrol. Some results indicate that stilboestrol added to glucose-phosphate dilutor increases the tendency to agglutination of the sperms of treated animals. Some other chemical substances have, however, the same effect, and stilboestrol added to dilutor has no effect upon the sperms of untreated animals.

SUMMARY

1. The sperm production and sex drive of two Suffolk rams were studied before and after implantation of diethylstilboestrol.

2. Sperm production increased 7–9 days after implantation and the effect lasted for about 5 days. The higher the dosage the higher the sperm production.

3. There was no effect on sex drive, sperm morphology or quality of spermatozoa.

4. Spermatozoa collected from treated animals showed a high tendency to agglutinate.

5. Sperms collected from stilboestrol-treated animals were fertile. Eight normal lambs (5 males and 3 females) were produced from 5 artificially-inseminated ewes.

6. The absorption rate of stilboestrol was 4·25–5 mg./day.

7. It is suggested that the effect on sperm production may be brought about by stimulation of the hypophysis to release secretion.

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MAINTENANCE AND RESTORATION OF GROWTH IN THYROIDECTOMIZED RATS

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The function of the thyroid glands in regulating the process of body growth was recognized many years ago from observations on human cretins. Subsequently, it was established that thyroidectomy in experimental animals checks the rate of growth, the retardation being greater when the operation is performed very early in life. Though these facts had long been established on a qualitative basis evidence of sufficiently quantitative precision is best available from studies of more recent years. Thus, Salmon [1938] found that the maximal body weight attained by rats thyroidectomized at birth was only 25–35 g.

The rate of growth in thyroidectomized animals can be maintained or restored to normal by the administration of various thyroid preparations, such as thyroid gland substance [Smith, Greenwood & Foster, 1927; Salmon, 1938], thyroxine [Evans, Simpson & Pencharz, 1939] or artificial thyroprotein prepared by iodination of the proteins of skimmed milk [Reineke & Turner, 1941]. According to Flower & Evans [1924] and Evans *et al.* [1939] the administration of extracts of anterior pituitary gland will also stimulate growth in thyroidectomized rats, although Salmon [1938] failed to restore growth in similar rats by the daily implantation of whole pituitary glands from adult rats. On the other hand, the dwarfing which normally follows removal of the pituitary gland cannot be prevented by the administration of thyroid gland preparations [Smith *et al.*, 1927] or by the injection of thyroxine [Evans *et al.*, 1939].

The exact relationship between the thyroid and the pituitary glands in controlling growth has not yet been elucidated. Smith [1933] showed that greater growth, accompanied by increased skeletal development, was produced in thyroidectomized-hypophysectomized rats when thyroid gland substance was administered in addition to pituitary gland extract, and Evans *et al.* [1939] believe that the secretions of the two glands act synergistically.

The results described below agree with much of the earlier work and, furthermore, show that crystalline thyroxine, implanted subcutaneously in tablet form by the technique of Deanesly & Parkes [1937], will maintain the growth rate of thyroidectomized rats at the level of that of intact rats.

MATERIAL AND METHODS

Animals used

Young male rats, having a body weight of about 40–50 g., were used. Thyroidectomy was carried out under 'Avertin' anaesthesia. The operation consists of separating the two lateral lobes of the gland by dividing the isthmus and removing each half in-

dependently by blunt dissection. Some haemorrhage occurs as the gland is being removed, but it is easily arrested by gentle pressure with swabs. Care is required in avoiding the recurrent laryngeal nerve.

Substances used

The effect of the following preparations was examined:

(*a*) 'Thyroids U.S.P. Desiccated' (Armour and Co.) stated to contain 0·2% iodine in thyroid combination; this was a very old preparation.

(*b*) Synthetic crystalline thyroxine ('Roche') and thyroxine and sodium thyroxine (B.D.H.).

(*c*) Acetone-dried pituitary glands of adult male rats.

(*d*) An extract prepared from the anterior-pituitary gland of oxen (AP52D).

(*e*) Mare serum gonadotrophin (PMS18).

(*f*) Chorionic gonadotrophin (UP31).

Observations on growth

These were made on all rats by once- or twice-weekly weighings at a constant time before feeding. At autopsy, the alimentary and reproductive tracts, spleen, liver, kidneys, heart, lungs and skin were weighed in the fresh condition. The remainder of the carcass after weighing was boiled and the skeleton prepared and weighed. The difference between the weights of the dissected carcass and of the skeleton was assumed to represent the weight of the total musculature. The reproductive tract and the adrenal glands were fixed in Bouin's fluid, and the constituent organs of the former were weighed subsequently on a torsion balance.

RESULTS

The effect of thyroidectomy on body growth

Eighteen intact and ten thyroidectomized male rats, having average body weights of 40 and 43 g. respectively, were weighed once weekly for 11 weeks. The results obtained are plotted in Fig. 1 (curves I and IV). At the end of the experiment the difference in average body weight between the two groups was 120 g. The weight of the thyroidectomized rats reached a maximum (80 g.) 6 weeks after operation and thereafter tended to fall in keeping with a marked deterioration in the condition of the animals. Symptoms of hypothyroidism, such as dry skin and staring hair, became evident.

Restoration of growth after thyroidectomy

Seven groups, each consisting of five thyroidectomized rats, were left without further treatment for 9–10 weeks until growth ceased. The substances listed in Table 1 were then given for 5 weeks by daily subcutaneous injections. All the rats were weighed once weekly from the time of operation until they were killed, with the exception of the rats in group 7 which were weighed twice a week.

The most satisfactory result was obtained with the thyroid gland preparation (Table 1, group 7): the growth curve of these rats is plotted in Fig. 1 (curve III). The average increase in weight of these rats was 50 g. From the day following the operation, however, these rats had been injected daily with 2 mg. of the acetone-dried powder from the pituitary glands of adult male rats. This amount did not maintain

growth at the normal rate, but twice the amount did cause a small but definite increase in the weight of rats already dwarfed by thyroidectomy (Table 1, group 3).

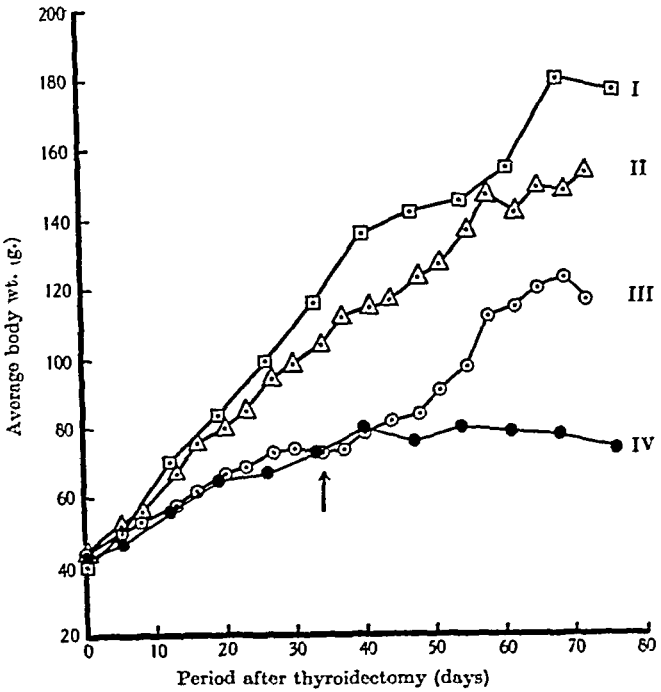


FIG. 1. The effect of thyroidectomy on body growth, and maintenance and restoration of growth with dried thyroid gland. ⊡ = Intact male rats (curve I). △ = Maintenance of growth with dried thyroid gland (curve II). ⊙ = Restoration of growth with the same thyroid gland preparation; the arrow indicates the day on which injections were commenced (curve III). ● = Thyroidectomized rats (curve IV).

Table 1. *Restoration of body growth in thyroidectomized rats. The amount of substance injected into groups 3–6 was doubled during the course of treatment*

	Treatment		Body weight (g.)		
Group	Substance	Daily amount mg.	At operation	At beginning of treatment	Maximum attained
1	None, intact controls	—	40	153	193
2	None, thyroidectomized controls	—	43	79	79
3	Rat pituitary gland	2·0→4·0	40	68	97
4	Ox pituitary gland	2·0→4·0	41	63	78
5	Mare serum gonadotrophin	0·5→1·0	43	74	89
6	Chorionic gonadotrophin	1·0→2·0	42	70	79
7	Dried thyroid gland	1·0	44	73	123

Concurrently with the above groups of rats, another group of rats was injected with a large dose of thyroxine which proved so toxic that they all died before any prolonged observations could be made on body weight.

The other preparations, extracts of (i) ox pituitary gland containing large amounts of thyrotrophic hormone, (ii) mare serum gonadotrophin containing follicle-stimu-

lating hormone, and (iii) chorionic gonadotrophin containing luteinizing hormone produced only a slight increase in the body weight of the thyroidectomized rats, bu caused hypertrophy of the secondary reproductive organs (see Table 2).

Maintenance of growth after thyroidectomy

The rest of the experiments were confined to observations on the maintenance o growth after thyroidectomy, chiefly because of the high rate of mortality that occurred in the longer experiments on the restoration of growth. The dried thyroid preparation and thyroxine were used.

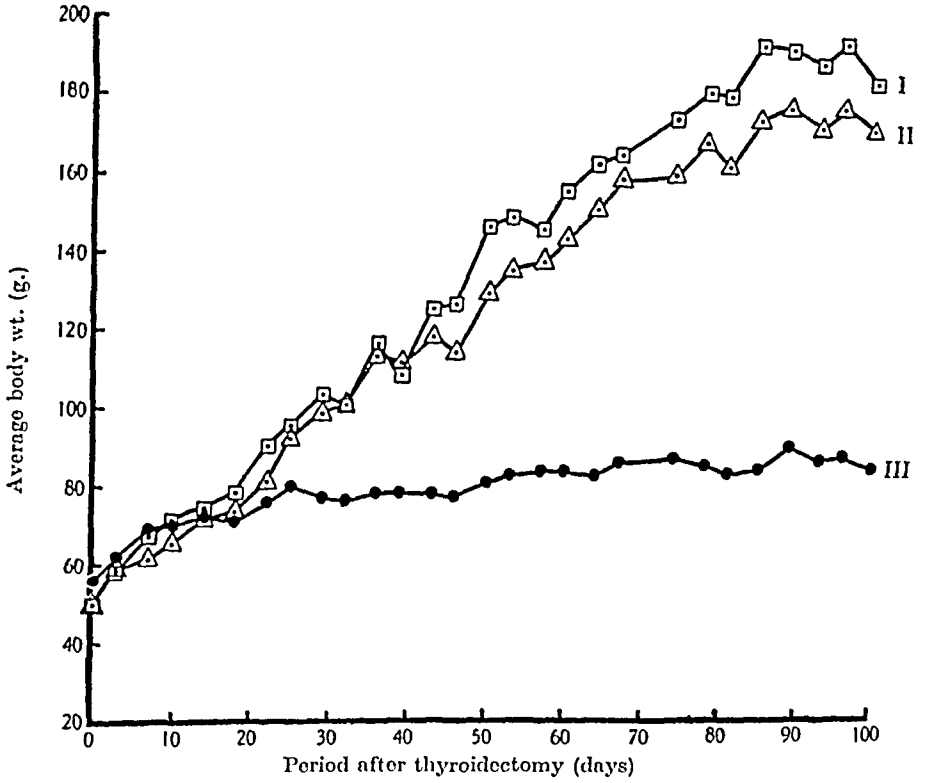


FIG. 2. Maintenance of growth in thyroidectomized rats by daily injection of thyroxine. ⊡ = controls, intact rats (curve I). ● = controls, thyroidectomized rats (curve III). △ = thyroidectomized rats injected daily with 2·5 μg. thyroxine (curve II).

Dried thyroid gland

Five rats having an average body weight of 45 g. were thyroidectomized and, commencing on the next day, they were injected daily for 11 weeks with 1 mg. of dried thyroid gland suspended in 1 ml. of water. They were weighed twice weekly; the average weights are plotted in Fig. 1 (curve II). An almost normal rate of growth was maintained in this way. The maximum weight (153 g.) was attained after 72 days. Throughout the experiment the rats remained in excellent physical condition, with the skin moist and the hair sleek and silky.

Thyroxine

The content of thyroxine-iodine in the dried thyroid powder was determined, and it was calculated that 1·5 μg. of thyroxine would be equivalent to 1 mg. of the

powder. Two sets of experiments were carried out on the administration of crystalline thyroxine.

(*a*) *Daily subcutaneous injection of thyroxine in aqueous solution.* Five thyroidectomized rats were injected daily with 2·5 μg. of crystalline thyroxine ('Roche') for 100 days, and weighed twice weekly. The average weights are given in Fig. 2 (curve II) together with those for a group of six untreated thyroidectomized rats (curve III), and five intact rats (curve I). It will be seen that the dose of thyroxine was adequate for the maintenance of normal body growth in the earlier period of treatment, but that later the rate of growth fell slightly below that of the normal rats.

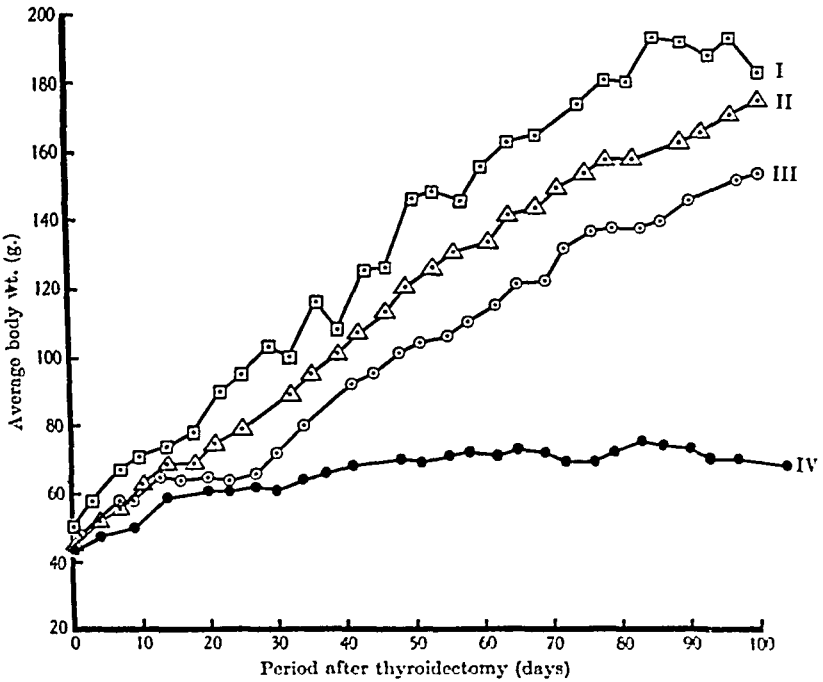


FIG. 3. Maintenance of growth in thyroidectomized rats by the implantation of tablets of thyroxine. □ = controls, intact rats (curve I). ● = controls, thyroidectomized rats (curve IV). △ = thyroidectomized rats implanted with tablets of thyroxine (curve II). ⊙ = thyroidectomized rats implanted with tablets of sodium thyroxine and cholesterol (curve III).

(*b*) *Subcutaneous implantation of crystalline thyroxine.* One tablet of 40 mg. of crystalline thyroxine (B.D.H.) was implanted subcutaneously into each of a group of ten rats at the time of thyroidectomy, no further treatment being given; seven of the rats survived for 100 days. The animals were weighed twice weekly. The average body weight of the seven rats, which at the time of thyroidectomy and implantation was 45 g., increased steadily throughout the experimental period until, at the end, it had risen to 174 g. (Fig. 3, curve II). The rate of growth was similar to that of the rats injected daily with 2·5 μg. of thyroxine, and was only slightly less than that of the intact control rats (curve I).

Into each of another ten rats at the time of thyroidectomy was implanted a 40 mg. tablet containing 40% sodium thyroxine and 60% of cholesterol as

excipient.[1] Only four of these rats survived the 100-day period; their average body weights are graphed in Fig. 3 (curve III). The rate of growth during the second fortnight was poor, but subsequently it steadily increased again. At the end of the experimental period the average body weight was 153 g., appreciably lower than that of the rats implanted with pure thyroxine. Of the rats in this group that died during the experiment, one, that lived for 97 days, had at no time a body weight greater than 72 g.; another, that died on the 76th day, had a body weight of 91 g. The growth rate of the former rat seemed to have been completely unaffected by the presence of the tablet. In general, therefore, the sodium thyroxine-cholesterol tablets were less satisfactory than those of thyroxine alone.

The tablets, when removed, were firmly encapsulated in a sheath of fibrous tissue; they were hard to the touch and showed no signs of disintegration, though Parkes [1942] does not find that tablets of pure thyroxine without excipient are so stable in intact rats and rabbits.[2] Neither the thyroxine nor the sodium-thyroxine tablets, when dried and weighed, showed any detectable loss in weight as the result of 100 days of implantation.

Effect of thyroidectomy on organ growth

The weights of a number of different organs at death showed that the retardation of growth after thyroidectomy was common to all.

Table 2. *Effect of thyroidectomy on the growth of the reproductive organs and adrenal glands of the rat, and the effects of various gonadotrophic preparations, dried thyroid gland and thyroxine*

		Substance injected		Body weight (g.)		Weight of organs (g.)			
Group	No.	Extract	Daily amount	Initial	At autopsy	Testes	Sem. ves.	Prostate	Adrenals
1	18	[Controls—intact]	—	40	193	2·300	0·306	0·400	0·030
2	10	[Controls—thyroidecto-mized]	—	43	73	1·480	0·053	0·150	0·020
3	10	[Controls—intact]	—	140	255	2·710	0·410	0·585	0·036
4	10	[Controls—thyroidecto-mized]	—	145	136	1·700	0·116	0·223	0·024
5	10	[Controls—intact]	—	—	147	1·190	0·041	0·114	0·026
6	5	Rat pituitary gland	4 mg.	40	96	2·190	1·050	0·770	0·020
7	5	Ox pituitary gland	4 mg.	41	77	1·660	0·224	0·326	0·017
8	5	Mare serum gonadotrophin (PMS 18)	1 mg.	43	89	2·270	1·540	1·110	0·020
9	4	Chorionic gonadotrophin (UP 31)	2 mg.	42	79	1·550	0·780	0·590	0·023
10	5	Dried thyroid gland	1 mg.	45	153	1·890	0·170	0·296	0·037
11	5	Thyroxine	2·5 μg.	50	176	1·940	0·325	0·351	0·028
12	7	Thyroxine	Tablet	45	174	2·250	0·384	0·291	0·025

The weights of the reproductive organs and of the adrenal glands, after fixation in Bouin's fluid, of most of the thyroidectomized and control rats used in the above experiments are given in Table 2. Thyroidectomy in immature rats (group 2) caused

[1] Sodium thyroxine, at least of this particular batch, was found not to make stable tablets without excipient.

[2] In the experiments recorded by Dr Parkes implantation was made into the flank, and it now seems likely that the better preservation of the tablets in the thyroidectomized rats was due to implantation being made into the neck.

some retardation in the growth of testis, but its effect on the accessory reproductive organs, especially the seminal vesicles, was greater. The growth of the reproductive organs of rats thyroidectomized at a time when the growth rate of these organs is very rapid was also retarded (cf. groups 3 and 4), but some growth had occurred after the operation, since younger intact rats of about the same body weight (group 5) had considerably smaller organs. These results confirm the observations of Smelser [1939].

The secondary reproductive organs of thyroidectomized rats injected with gonadotrophic preparations were greatly enlarged. The seminal vesicles of those injected with mare serum gonadotrophin (group 8) for instance, were about thirty times the size of those of the thyroidectomized controls (group 2) and five times the size of those of the intact controls (group 1). The organs of the rats treated with thyroxine, on the other hand, are maintained at normal size.

Growth of the adrenal glands was also retarded by thyroidectomy, and can be restored by injection of desiccated thyroid or thyroxine and to a lesser extent by the implantation of a tablet of thyroxine.

DISCUSSION

From the results described above and from those of Salmon [1938] it appears that the degree of retardation of growth following thyroidectomy in the pre-pubertal rat depends on the weight of the animal at operation. Salmon thyroidectomized newborn rats and found that they ceased growing when they reached 25–35 g. If the operation is performed on rats weighing about 40 g. growth ceases at about 80 g. On the other hand, rats thyroidectomized after maturity (140–150 g.) lost, on an average, 10 g. body weight over a period of 12 weeks.

There are several points of resemblance between the effects of thyroidectomy and of hypophysectomy. Retardation of body growth is a characteristic effect of both these operations and, moreover, the maximum weights attained by rats, when subjected to either operation at 40 g., are similar. Again, the effects on the reproductive organs are of the same kind, although those of hypophysectomy are undoubtedly more severe; whilst thyroidectomy causes merely a retardation in growth, hypophysectomy results in atrophy of these organs. Finally, the physical appearance and condition of the rats, the dryness of the skin and the coarseness of the hair, are similar after both operations. Whether these effects are caused directly and independently, or are indirectly co-ordinated by the action of these glands on each other, is uncertain.

An almost normal rate of growth can be maintained in thyroidectomized rats by the administration of dried thyroid substance or of crystalline thyroxine injected in aqueous solution, or implanted subcutaneously in the form of a tablet. The fact that a daily dose of 2·5 μg. of thyroxine by injection seemed adequate agrees well with the results reported by Evans *et al.* [1939], who maintained body growth and basal metabolism at normal levels in thyroidectomized rats about twice the size, on 5·0 μg. of thyroxine daily. From observations on the restoration of a normal heart rate in thyroidectomized rats, however, Fishbourne & Cunningham [1938] consider that the output of thyroxine by the thyroid gland is about 40 μg. daily.

The maintenance of body growth by a tablet of sodium thyroxine, with cholesterol as an excipient, was not so complete as that obtained with thyroxine alone, in spite of the fact that the sodium compound, on account of its greater solubility, might have

been absorbed more readily. The absence of any appreciable loss in weight from either type of tablet excludes the possibility that the rats received thyroxine in supra optimal dosage.

Even allowing for the errors inherent in detecting small losses from large tablets after prolonged implantation, it seems very unlikely that the tablets could have lost as much as an average of 1 mg. in the 100 days. From the experiment on the injection of dried thyroid in suspension or of thyroxine in solution it would appear that the tablets need only have lost 1·5–2·5 μg. per day, or 0·25 mg. (250 μg.) throughout the whole period of 100 days, to have produced the observed effect. A single tablet of 50 mg. of thyroxine, therefore, should maintain growth in a thyroidectomized rat for about 50–60 years! I am unable to confirm Wokes's [1941] observation that a tablet of thyroxine, implanted subcutaneously, in normal animals, exerts its effect for only a limited period of time.

In the case of steroid hormones there has been no difficulty in adapting the technique of implantation to clinical use, and it has proved successful for the prolonged administration of desoxycorticosterone, testosterone and oestrone. It would seem, therefore, that a clinical trial of this technique for the administration of thyroxine in cases of thyroid deficiency would be well justified, and Wokes's [1941] warning, based on the production of hyperthyroidism in normal guinea-pigs by the implantation of crystalline thyroxine, need not be assumed, without such trial, to be applicable to man in a state of hypothyroidism.

SUMMARY

1. Quantitative observations have been made on the growth rate of thyroidectomized rats, and attention is drawn to some similarities between the effects of thyroidectomy and hypophysectomy.

2. The retardation of growth following thyroidectomy can be overcome by injections of desiccated thyroid gland or thyroxine, or by the implantation of a tablet of thyroxine; it cannot be overcome by the injection of rat pituitary gland or of various gonadotrophic or thyrotrophic substances in the dosages used.

3. It is calculated that the rate of absorption of a 40 mg. tablet of thyroxine, implanted subcutaneously, which is sufficient to promote normal growth in a thyroidectomized rat, is rather less than 1 mg. per annum.

Acknowledgement is made to Dr F. G. Young who prepared the extract of ox pituitary gland (AP 52); to Organon Laboratories, Ltd., for the chorionic gonadotrophin (UP 31), and to the Løvens Kemiske Fabrik for the mare serum gonadotrophin (PMS 18).

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GAMETOGENESIS AND SOME ENDOCRINE FACTORS AFFECTING IT IN THE ADULT MINNOW (*PHOXINUS LAEVIS* L.)

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Descriptions have been given [Bullough, 1939] of the normal reproductive cycles of male and female minnows (*Phoxinus laevis* L.), and the main outlines of spermatogenesis and oogenesis in this fish are known. Interest has recently been focused on the source of new oogonia in the ovaries of adult vertebrates, and a considerable amount of evidence [Allen, 1923; Swezy, 1933; Bullough & Gibbs, 1941] has been brought forward which leaves little doubt that, in many mammals and at least one bird, new crops of eggs are produced cyclically throughout adult life from the germinal epithelium investing the ovary. Evidence of similar conditions in the female minnow has now been obtained, and this is recorded below.

Considerable interest has also centred round the environmental and endocrine mechanisms underlying reproductive cycles, and work on these lines in the minnow has been done by Bullough [1939, 1940] and by Spaul & Bullough [unpublished]. The main factors influencing gametogenesis are generally well known, and certainly in many cases the ultimate contróls are the seasonal variations in the length of day and the induced secretions of the anterior pituitary gland. Injection experiments with the minnow, recorded here, have now indicated that the control of at least some phases of gametogenesis is probably more complicated than has been supposed.

MATERIAL AND METHODS

For the study of the normal reproductive cycles of male and female minnows, fish were obtained from Lake Windermere at various times of the year from July 1936 to the following breeding season. They were usually killed and fixed shortly after their arrival in Leeds. The minnows used in the experimental work came from Lake Windermere in July 1939, and were kept in the laboratory in white enamelled metal tanks (40 × 25 × 30 cm. deep). Each tank held about forty fish which were fed on ant pupae. The water was changed daily, and in these conditions very few deaths occurred.

The experimental work consisted of a series of injections of male and female sex hormones into minnows which had recently spawned. The male sex hormone used was testosterone propionate (Perandren, Ciba, Ltd.) diluted ten times with sesame oil. After dilution the solution contained 0·5 mg. of testosterone propionate per ml. The female sex hormone, oestrone (Menformon, Organon Laboratories), was diluted ten times with Ringer solution, and the concentration was then 0·01 mg. (100 i.u.) of oestrone per ml. In all the experiments 0·2 ml. of the diluted hormone solutions was

injected into the body cavity of each fish at each injection. The following experimental groups were set up on 27 July:

(1) Control group of fifteen female and fifteen male fish killed on 27 July at the start of the experiments.

(2) Control group of fifty female fish which were not injected.

(3) Control group of fifty female fish injected with Ringer's solution.

(4) Group of fifty female fish injected with oestrone in Ringer's solution.

(5) Control group of fifty male fish which were not injected.

(6) Control group of fifty male fish injected with sesame oil.

(7) Group of fifty male fish injected with testosterone propionate in sesame oil.

On 28 July the first injections were given, and these were repeated every third day. Fifteen fish from each group were killed on 18 August after they had received seven injections, and a second sample of about fourteen fish from each group was taken on 8 September after fourteen injections. Finally, after twenty-one injections, the remaining fish, about thirteen in each group, were killed on 29 September. The water remained throughout at the normal summer tap-water temperature. It varied between 14 and 18° C. with an average of about 16·5° C. In no group was the mortality unduly high, and all the fish continued to feed actively. In those fish which received injections of watery solutions, the wounds due to the hypodermic needle healed rapidly, but in those injected with oily solutions there was abdominal distension and healing was much slower. In these latter fish at the end of the experiments the wounds ceased to heal properly, but even this did not cause any great mortality.

All the fish were killed by a quick incision through the spinal cord just behind the skull, and after the body cavity was opened they were fixed whole for 2 days in Bouin's fluid before being transferred to 70 % alcohol. The reproductive systems were then dissected out, and transverse and longitudinal sections of the gonads and gonoducts were cut to a thickness of 7 μ. The sections were stained either in Heidenhain's iron haematoxylin and van Gieson's stain, or in Mayer's haemalum and orange G.

OBSERVATIONS

Female minnows

Normal oogenesis

After the spawning season the production of new oogonia proceeds rapidly in the ovary of the minnow, and during the summer months many oogonia grow and are transformed into primary oocytes of the primary growth phase. A few larger primary oocytes of the secondary growth phase, probably left over from the previous year's cycle of maturation, are also present, and there are masses of old follicle cells which are homologous with the corpora lutea of the mammals. In early autumn there is a great increase in the size of the ovary due to the rapid production and growth of primary oocytes of the primary and early secondary growth phases. Little further change takes place during the late autumn and winter, and the final growth and maturation of the oocytes begins in early spring. A more detailed description of these processes has been given by Bullough [1939].

The mode of origin of the new oogonia, which are formed in the ovary of the minnow in early summer, was stated by Bullough to be uncertain, and the possibility was suggested that, as is reported in the cases of *Pleuronectes limanda* L. [Wheeler, 1924] and *Gasterosteus aculeatus* L. [Craig-Bennett, 1930], some of the old follicle cells

of the previous year give rise by direct growth to the new supply of oogonia. It has now been found that this supposition was incorrect, and the true mode of origin of the new oogonia has been determined. The ovary of the minnow is bounded externally by a very thin connective tissue sheath, the tunica albuginea, and immediately outside this sheath are isolated groups of cells which form a discontinuous germinal epithelium. In the distended pre-spawning ovary in early spring the patches of germinal epithelial cells are tightly stretched and widely separated, and the cells are best observed in the smaller post-spawning ovary. Here they are seen to be roughly cubical in form with a nuclear diameter of about 9 μ. The tunica albuginea and germinal epithelium are normally thrown into deep folds which extend down into the centre of the ovary between the developing oocytes, and at the hilus of the ovary the patches of germinal epithelium are continuous with the peritoneum. In post-spawning ovaries in July and August, many cells of the germinal epithelium were found to be actively dividing. It is probable that these mitotic divisions sometimes result in the production of new cells of the germinal epithelium, but it was also clearly apparent that they frequently result in the production of new oogonia which pass through the tunica albuginea and remain attached to it on the inner surface. It is also possible that some of the mitoses result in the production of follicle cells, as each new oogonium has two or three of these cells in close association as soon as it is formed. It was observed that the cells of each patch of germinal epithelium often divided about the same time (Plate 1, fig. 2), and gave rise to a group of five or six young oogonia together with their follicle cells. The subsequent growth of the group of new oogonia was not necessarily synchronous, and usually one or two of them grew much more rapidly than the others (Plate 1, fig. 1). These groups of small oogonia were very common in the ovaries of fish taken in August, and even at the end of that month mitoses of the germinal epithelial cells were observed. As the germinal epithelium commonly extended into the centre of the ovary, the mitoses and the groups of young oogonia were present not only on the surface but throughout the whole organ.

Towards the end of August the numbers of mitoses observed became rapidly less, and in early autumn a great increase in the size of the ovary took place due to the rapid growth of the oogonia and young primary oocytes. Thereafter, throughout late autumn and winter, very small oogonia were not commonly seen, and no mitoses of the germinal epithelium were observed. It appeared that the production of new oogonia, if it did not entirely cease, slowed considerably. During the period of rapid growth of the ovary in early spring, young oogonia were still uncommon, and a lack of activity of the germinal epithelium of actively growing ovaries was also noticed in minnows subjected to extra light and warm conditions in winter. When the ovaries were fully developed, however, the active production of new oogonia from the cells of the germinal epithelium started once more. Whether the rate of production of new oogonia is as active at this time as it is in the post-spawning ovary was difficult to determine owing to the great size of the pre-spawning ovary and the consequent wide separation of the patches of germinal epithelium. The conclusion was, however, reached that the peak of activity of the germinal epithelial cells does not occur until just after spawning is completed.

Experimental investigations

At the beginning of the injection experiments, in late July, the ovaries were in the small post-spawning condition. The germinal epithelia were active, and there were numbers of oogonia and primary oocytes of the primary and early secondary growth phases as described above, as well as several small masses of old follicle cells, the 'corpora lutea'. This is the normal appearance of the minnow's ovary at this time of year, and apart from the continued production of new oogonia and the partial disappearance of the 'corpora lutea', there was little variation from this general structure in the ovaries of the control fish killed on 18 August. By 8 and 29 September the activity of the germinal epithelium appeared to have almost ceased, and therefore few very young oogonia were in evidence. The 'corpora lutea' had nearly all disappeared. The ovaries of the fish injected for control purposes with Ringer's solution were indistinguishable from those of the normal controls.

In the fish injected with oestrone in Ringer's solution the ovaries showed immediate changes. After only seven injections the larger primary oocytes in the secondary growth phase showed disintegrative changes which included vacuolation and loss of structure of the cytoplasm, yolk, and nucleus. After fourteen injections many oocytes were reduced to homogeneous lightly staining masses, still, however, surrounded by the vitelline membrane. This membrane became thinner, and finally, as it broke down, the remnant of each oocyte was invaded by masses of small follicle cells. After twenty-one injections the breakdown of many of the largest oocytes was completed, and all the primary oocytes in the secondary growth phase were in some stage of breakdown. None of the primary oocytes of the primary growth phase and none of the oogonia were affected in this way, and they all appeared normal.

The breakdown of the large primary oocytes was the most obvious effect produced by the oestrone, but there was a second, and more important, effect. The injections induced great mitotic activity in the cells of the germinal epithelium, and this had two results. First, the number of germinal epithelial cells increased. As a consequence the patches of germinal epithelium were more extensive, and over considerable areas they became confluent. This joining of the patches was also helped by the shrinkage of the surface of the ovary as the large primary oocytes broke down. The second result of the abnormal mitotic activity of the germinal epithelial cells under the influence of oestrone was the production of very large numbers of small oogonia. The germinal epithelium was so extensive and the number of oogonia produced so great that, instead of being present in small groups as in the normal ovaries (Plate 1, fig. 1), the oogonia were seen in the sections either in big masses of twenty or thirty (Plate 1, fig. 3) or in long rows. In the solid ovary these oogonia must often have been present as extensive sheets of cells lying just under the tunica albuginea. Both these effects, the increase in the numbers of germinal epithelial cells and of young oogonia, were evident after seven injections of oestrone, but the full response was not apparent until September in those fish given fourteen and twenty-one injections. This is brought out in Table 1, which shows the average number of oogonia present in twenty similar longitudinal sections of one ovary of each of the experimental groups. Each figure represents the average of counts made on one of the ovaries from each of ten individuals. Because of the size of many of the oogonia, the same cell was often

Table 1. *Numbers of oogonia present in twenty median longitudinal sections of an ovary from the control and oestrone-injected groups. Each figure represents the average of counts from ten ovaries*

Date	Control	Oestrone-injected
27 July	1,266	—
18 Aug.	1,720	4,312
8 Sept.	3,269	11,216
29 Sept.	2,023	14,067

represented on several successive sections. The counts were therefore made on every third section. It is seen that in the control minnows during September the number of oogonia decreased due to the fact that the stock was being drawn upon for the formation of primary oocytes while no new oogonia were being formed. Even at the end of that month, however, the epithelial cells of the oestrone-injected minnows were still actively undergoing mitosis and producing thousands of new oogonia.

Male minnows

Normal spermatogenesis

The normal yearly cycle of spermatogenesis in the adult minnow has been described in detail by Bullough [1939]. Briefly it may be summarized as follows. After the spawning season in June and early July, there is a period of reconstruction within the testis, and during the summer some new cysts of spermatogonia are slowly produced by the mitotic divisions of a few of the primary germ cells. These processes are greatly accelerated in autumn, and by the end of October the first primary spermatocytes appear. Little change takes place in the colder months, but in early spring the production of new spermatogonia slackens, while that of the secondary spermatocytes, spermatids, and spermatozoa proceeds rapidly. Fish taken in early June possess testes and vasa deferentia distended with great masses of spermatozoa.

Experimental investigations

The testes of the control minnows killed in late July were in the normal post-spawning condition. The processes of reconstruction had just started, and in the thread-like testes divisions of the primary germ cells and spermatogonia were slowly proceeding. In this way small cysts of spermatogonia were established within the lobules, and these processes continued slowly during the course of the experiments. The control fish taken on 18 August and 8 September differed little, and a section of the testis of a minnow in late July (Plate 2, fig. 4) could represent them equally well. By 29 September the rate of production of the spermatogonia had quickened (Plate 2, fig. 5), and the testes were slightly enlarged. The testes of minnows injected with sesame oil were normal.

In those fish injected with testosterone propionate in sesame oil there was immediately some stimulation of those mitotic divisions which had been slowly taking place within the testis. After seven injections mitoses were relatively common, and in a few individuals some primary spermatocytes had appeared. As a consequence the testes were enlarged. After fourteen injections considerable changes had occurred, and primary and secondary spermatocytes, spermatids and spermatozoa were common. The spermatozoa had been immediately released from the testis, and they were present in large numbers in the vas deferens which had itself acquired an opening

to the exterior. Complete spermatogenesis had been induced, but the already exhausted post-spawning testis only produced a small fraction of the spermatozoa normally present in the breeding season. The testes did not swell up, and as the spermatozoa passed from them into the vasa deferentia, they were greatly reduced in size. After twenty-one injections the effects were even further accentuated. More and more spermatozoa had been produced, had left the testis and had been shed to the exterior. The organ was then extremely thin and small. It contained all cell stages from a primary germ cell to a fully developed spermatozoon (Plate 2, fig. 6), but a very large number of the cells present formed a resting reserve of primary germ cells and spermatogonia which were apparently unaffected by the treatment.

Table 2. *Proportion of different cell types in the testes of control and testosterone-treated minnows*

Cell type	27 July %	18 Aug. %	8 Sept. %	29 Sept. %
(*a*) *Control minnows*				
Primary germ cells	35	22	19	18
Spermatogonia	65	78	81	82
Other types	0	0	0	0
(*b*) *Minnows injected with testosterone propionate*				
Primary germ cells	—	10	7	2
Spermatogonia	—	82	20	9
Spermatocytes	—	8	45	16
Spermatids	—	0	9	4
Spermatozoa	—	0	19	69

Table 2 illustrates these results. Estimations were made of the relative proportions of the different cell types in the testes from control and injected minnows. In each case the estimations were made on comparable areas of the longitudinal sections of ten testes, over 10,000 cells being counted for each estimation.

DISCUSSION

The observations and experiments recorded here demonstrate the source of new oogonia in the ovary of the minnow and the effect of the sex hormones on gametogenesis. Oestrone is shown to stimulate the mitotic activity of the germinal epithelium with resulting production of new oogonia, and the testosterone propionate induces complete spermatogenesis in the testis. These results have been verified by Spaul & Bullough (unpublished) who performed similar experiments in late autumn and early winter. In similar conditions to those described here, the same responses were obtained to injections of the two sex hormones. The only difference was the quicker completion of spermatogenesis. Full summer recovery having taken place, the testes were stocked with masses of spermatogonia and primary spermatocytes so that the organ was better able to respond to the treatment. Complete spermatogenesis was achieved after only nine injections. Even at this time of year, however, not nearly so many spermatozoa were produced as in the normal breeding season, and at the end of the experiments, when most of the spermatozoa had passed into the vasa deferentia, the testes were thin and small. One important point demonstrated by these injections in autumn and winter was the effect of temperature. Bullough [1939] showed that a certain minimum temperature, about 10° C., is necessary before

minnows will respond by full maturation to extra light in winter, and Spaul & Bullough also showed that the same minimum temperature is necessary before they can respond to the sex hormones. The gonads of injected minnows kept at 9° C. showed little or no change.

Sex hormones have been injected into normal animals of many other species, although the effects of the female sex hormone on the ovary do not appear to have been so frequently examined in detail as the effects of the male sex hormone on the testis. A review of the literature concerning the effect of oestrogens on the female has been made by Allen, Hisaw & Gardner [1939], who reached the general conclusion that, at least in the mammals, such treatment generally has an inhibitory or depressing effect on follicular development. Sterility and abortion are produced. Nobody appears to have noticed any effect on the cells of the germinal epithelium, a fact perhaps due to the general lack of belief that these cells do have cyclic phases of activity during which they produce new crops of oogonia. Male sex hormone, when injected into normal male animals, has produced variable results. Wells & Moore [1936], using the ground squirrel *Citellus tridecemlineatus*, an animal which like the minnow has a pronounced winter anoestrus, have shown that injections of androsterone or testis tissue extract cause the appearance of spermatozoa precociously in young animals and unseasonably in adults. They also produced similar results with pregnancy urine and other gonadotropic agents such as fresh pituitary tissue. Wells & Gomez [1937] have also induced testicular activity in young and old, normal and hypophysectomized ground squirrels by treatment with androsterone or testosterone acetate, but against these results there is a considerable volume of evidence, summarized by Moore [1939], which demonstrates harmful effects of male sex hormones on the testis. In young rats testicular development is inhibited by injections of testis extracts or of androsterone or testosterone. Moore & Price [1932] have explained this by suggesting that an excess of oestrogen or androgen in the body inhibits the secretions of the anterior pituitary gland so that insufficient gonadotropin is produced for the stimulation of the normal development of the gonads. They therefore consider that the injurious effects on the testis are indirect. Moore & Price [1932] have also shown, however, that adult rats when injected with androgen show neither injury nor stimulation of the testes, while Cutuly & Cutuly [1940] have performed experiments and summarized evidence to show that injections of androgen induce and maintain spermatogenesis in hypophysectomized rats.

In spite of the frequent reports of injurious or indifferent effects it does appear certain that, when sex hormones are injected in the correct manner and in the correct doses, they can induce the nuclear changes associated with gametogenesis in both females and males. In the female the effect is shown in the stimulation of the first nuclear division of oogenesis, the division of the germinal epithelial cell to form the oogonium. The growth of the egg in the fish, like that of the follicle in the mammal, appears to depend for stimulation not on the oestrogens, which are shown to be injurious to these processes, but probably to the direct action of some secretion of the anterior pituitary gland. In the male, spermatogenesis is almost entirely concerned with cell divisions, either mitoses or meioses, and these are stimulated by androgens in the minnow, the ground squirrel and possibly the rat. It is interesting to note, however, that in the minnow large numbers of primary germ cells and sperma-

togonia were not affected by the male sex hormone. This raises the question of whether only a certain number of these resting reserve cells can be stimulated to develop each season, and whether the remainder, continuing dormant in a manner analogous to the pupation diapause of many insects, cannot be stimulated even by artificial treatment.

The tentative conclusion is reached that, at least in the minnow and the ground squirrel, the primary effect of the gonadotropic secretions of the anterior pituitary gland is to cause the secretion by the gonads of a sex hormone which in turn acts on the gonads and induces the nuclear divisions associated with gametogenesis. This theory would fit many known facts concerning the reproductive cycle of the minnow. In the female in early spring the period of growth of the eggs is not associated with the opening of the oviduct [Bullough, 1939], a happening which is controlled by oestrogen. During this period, therefore, it does not appear that the ovary produces any significant quantity of oestrogen, and, in consequence, no breakdown of the larger eggs takes place and no activity is observed in the cells of the germinal epithelium. Only at the very last moment before spawning does the oviduct acquire an opening to the exterior. At this time the germinal epithelium becomes active, and the final maturation divisions take place, themselves possibly induced by the sudden secretion of oestrogen. Not enough time is available for any destructive changes to appear in the large eggs before they are shed. It appears that oestrogen continues to be produced during the early summer causing the mitoses of the cells of the germinal epithelium and preventing the growth of eggs in the secondary growth phase of the primary oocyte. When this secretion ceases in late summer, the activity of the germinal epithelium also ceases, the larger eggs are again able to develop, and the autumn burst of ovary growth ensues. In the male there is a different timing in the production of the sex hormone. The opening of the vas deferens to the exterior and the development of the brilliant nuptial coloration take place at the same time as the rapid growth of the testis in early spring [Bullough, 1939], and it is probable that the maturation of the germ cells, like the development of the accessory and secondary sexual characters, is caused by androgen. The secretion of androgen does not appear to continue after the spawning season.

It is interesting to note the specificity of the stimulating effects of the two sex hormones. It was shown by Bullough [1940] that male sex hormone disintegrates the ovary of the minnow, and that the same effect is evident in the testis after injections of female sex hormone. Similar effects have been reported in such other animals as, for instance, rats [Golding & Ramirez, 1928] and sparrows [Ringoen, 1938]. Moore & Price [1932] have again explained this effect as due to the suppression of the anterior pituitary secretion because of the antagonism of the unusual quantities of sex hormone, but their theories may need some modification now that the specific stimulating effects of both oestrogen and androgen have been demonstrated.

SUMMARY

1. Short accounts are given of gametogenesis in the minnow (*Phoxinus laevis* L.), and a more detailed description is included of the method of formation of new oogonia. It is shown that new oogonia appear in late spring and early summer due to the mitotic activity of the cells of the germinal epithelium.

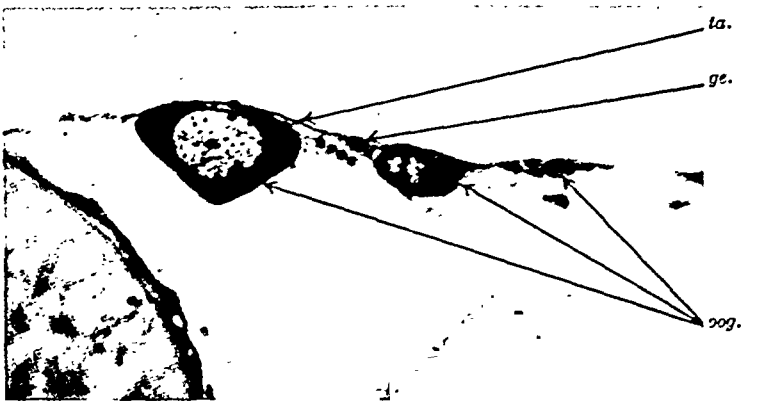


FIG. 1.

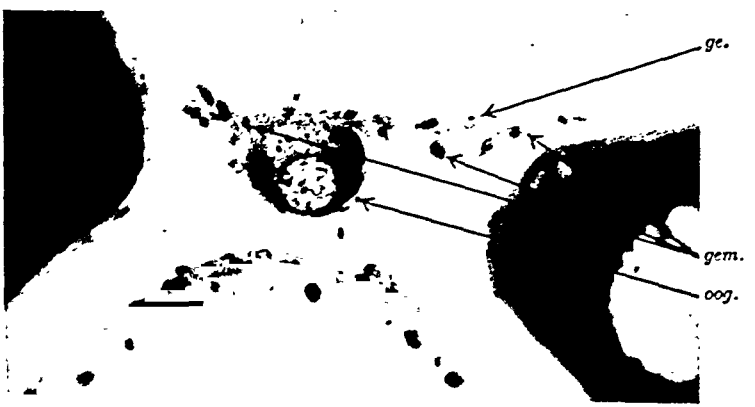


FIG. 2.



FIG. 3.

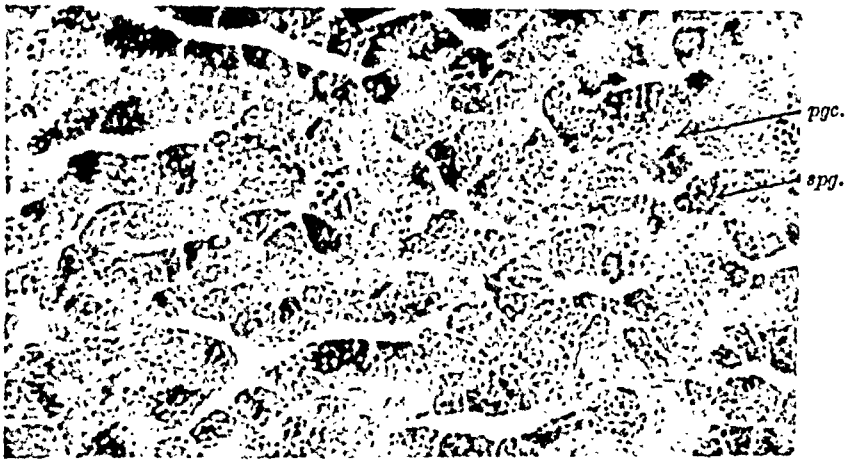


Fig. 4.

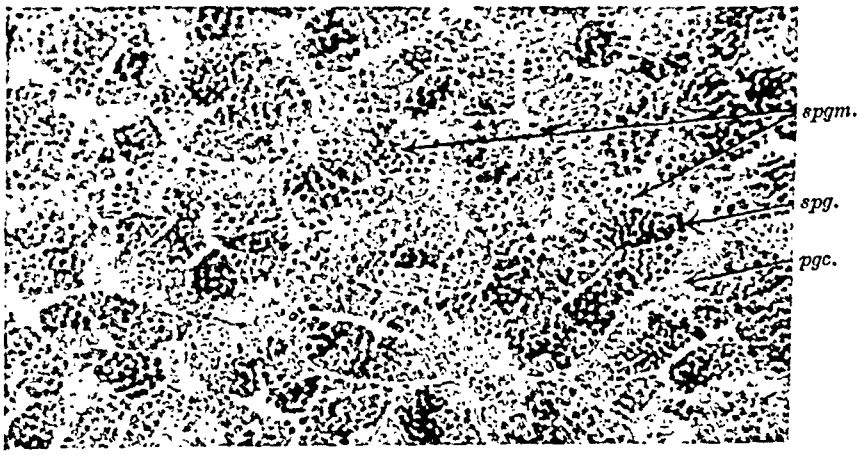


Fig. 5.

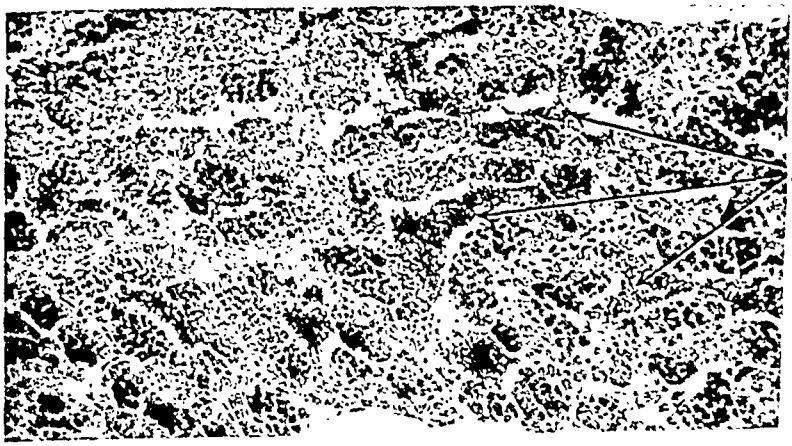


Fig. 6.

2. The effects of injections of oestrone on the post-spawning ovaries are described. Oestrone causes the breakdown of the primary oocytes in the secondary growth phase, and at the same time stimulates the mitotic activity of the germinal epithelial cells so that abnormally large numbers of young oogonia are produced.

3. The male sex hormone, testosterone propionate, causes mitosis and meiosis to take place rapidly in the post-spawning testis, and it is possible to induce full spermatogenesis with the development of large quantities of spermatozoa.

4. The conclusion is reached that, in the minnow, oestrogen stimulates at least one of the divisions of oogenesis, and that androgen stimulates all the spermatogenic divisions. Further, it is evident that the great growth of the primary oocyte, like that of the follicle in the mammal, is under some other control, probably the direct action of one of the hormones of the anterior pituitary gland. The suggestion is therefore made that, at least in some animals, the known action of the anterior pituitary gland in stimulating the nuclear divisions of gametogenesis is indirect, and is exerted through the intermediary of the sex hormones.

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EXPLANATION OF PLATE 1

FIG. 1. Group of three developing oogonia attached to the germinal epithelium of a minnow killed in August. ×500.

FIG. 2. Mitoses of the germinal epithelial cells of a minnow killed in July. ×500.

FIG. 3. Mass of newly-formed oogonia in the ovary of a minnow killed in September after 21 injections of oestrone. ×500.

Ge. germinal epithelial cell; *gem.* mitosis of germinal epithelial cell; *oog.* oogonium; *oogm.* mass of newly-formed oogonia; *ta.* tunica albuginea.

EXPLANATION OF PLATE 2

FIG. 4. Section of testis from a minnow killed late in July. ×250.

FIG. 5. Section of testis from a minnow killed late in September showing many newly-formed spermatogonia. ×250.

FIG. 6. Section of testis from a minnow killed in September after 21 injections of testosterone propionate. Complete spermatogenesis has been induced and masses of spermatozoa are present. ×250.

Pgc. primary germ cell; *spg.* spermatogonium; *spgm.* spermatogonia in mitosis; *sz.* spermatozoa.

Photomicrographs by Mr J. Manby, F.R.P.S., University of Leeds.

ADMINISTRATION OF NON-STEROID SUBSTANCES BY THE IMPLANTATION TECHNIQUE

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The subcutaneous implantation of compressed tablets of pure crystalline material has proved a highly successful method of administering steroid hormones. The essential feature of the technique, which was introduced by Deanesly & Parkes [1937], and which has given particularly favourable results with oestrone, oestradiol, progesterone, testosterone and desoxycorticosterone, is that it allows a very prolonged action for periods up to years from a single administration, and a very high effectiveness in relation to the total dose. The fact that months or years may be required for the complete absorption of a tablet depends on the low solubility of the steroid substances in body fluids, and it would be expected that the existing technique could be applied only to substances of this kind. However, attempts have recently been made to adapt the implantation technique to the administration of a variety of other substances, some of them having a much greater solubility in body fluids than the steroid hormones.

A study of the absorption of adrenaline from subcutaneously implanted tablets of the pure substance was made by Bottomley, Folley, Walker & Watson [1939], who used the method to investigate the effect on milk sugar of a prolonged rise in blood sugar. They implanted single tablets of adrenaline weighing about 50 mg. into goats, and tablets totalling 490 mg. into a heifer. There was an increase in blood sugar for nearly 2 days, and, with one exception, the animals showed no untoward local reaction. Similarly, Goetsch [1940] implanted tablets of adrenaline weighing about 100 mg. into rabbits and found clear physiological evidence of absorption, which was substantiated by the fact that after removal the tablets were found to have lost about 5 mg. of substance per day.

Parkes & Young [1939] investigated the possibility of administering insulin by the implantation of solid material. They found that 5 mg. pellets of either crystalline or low-zinc insulin, containing 100 units, were absorbed within 24 hr. The effect on the blood sugar was little different from that of a single subcutaneous injection. Mark & Biskind [1940] confirmed this result as regards crystalline zinc insulin, but further found that the addition of 20% of protamine to the zinc insulin pellet greatly prolonged the effect obtained by implantation. A more comprehensive experiment was carried out by Cutting, Morton & Cohen [1941] who made pellets of crystalline zinc insulin to which had been added various excipients, including cholesterol, which they found to be the most satisfactory.

A small amount of work has also been carried out on the administration of thyroxine in solid form. Greene & January [1940*a*] reported that slight absorption only took place from tablets of thyroxine implanted in dogs and men. Wokes [1941], however, obtained clear evidence of physiological effect by producing symptoms of

hyperthyroidism in normal guinea-pigs by the implantation of tablets of thyroxine. He found that the tablets had a very limited period of action, but this result is in sharp contrast to that obtained by Rowlands [1942].

Greene & January [1940*b*] carried out implantation experiments in the treatment of diabetes insipidus with posterior pituitary preparations. They were able to obtain a relatively prolonged anti-diuretic effect in dogs by the implantation of pellets made of compressed desiccated gland, but the technique was not clinically successful on account of the serious local reaction to the pellet.

The gonadotrophins, with the exception of that from mare serum, are typically substances which require to be administered daily or twice daily in order to obtain a maximum effect. Early in the work on chorionic gonadotrophin Shelesnyak & Engle [1932] investigated the possibility of giving the whole necessary dose in one administration by inserting a collodion sac containing the material into the subcutaneous space. Other experiments were carried out with compressed pills. The results were not notably satisfactory. Implantation of pituitary extract in powder form was tried by Loeser [1930] and by Jansen & Loeser [1930]. They obtained a definite biological response. More recently, Shuler [1941] has investigated the absorption of extracts of ox pituitary gland from tablets containing 25 mg. of the pituitary powder with about 20 % milk sugar. These workers obtained an effect as measured by increase of weight in the testes, ovaries or uteri of the experimental rats. The question of increasing the effectiveness of the extract does not seem to have entered into these experiments, but the observations are of considerable interest when considered in relation to the report of Meyer & McShan [1941] that very frequent injection greatly increases the maximum response obtainable with ox and sheep pituitary extracts.

The present paper describes an investigation into the possibility of adapting the implantation technique to the administration of non-steroid substances, with special reference to the use of excipients.

TECHNIQUE

Preparation of tablets

The excipient, where used, was mixed very carefully with the substance to be administered, and tablets varying from 10 to 250 mg. were made in a hand tablet-making press. By varying the size of the punches and dies a machine of this type, which is a standard article of commerce, can be used satisfactorily for almost any size of tablet, greater than 10 mg., required for this type of work, and there seems to be no justification for the various improvised punches and presses of which extensive accounts have appeared in the literature.

Implantation of tablets

Tablets were implanted into rabbits and rats, under ether anaesthesia, usually into the flank, and it has been found possible to insert as many as five 100 mg. tablets into one subcutaneous pocket in either species. All the experiments described below were performed on rats unless it is definitely stated that rabbits were used.

RESULTS

Excipients

In the case of highly water-soluble substances it was found that a tablet made without excipient was rapidly dissolved and absorbed by the subcutaneous tissue, and a search was therefore made for a suitable excipient, the slow absorption of which would continually free small amounts of the active substance. It was soon found, however, that a highly water-soluble substance was extracted from the tablet by the tissues even where the excipient was present in considerable excess and was itself not absorbed in weighable amounts. In the case of highly water-soluble substances, therefore, the criteria of a suitable excipient are that it should hold up the absorption of the substance as long as desirable, that it should not cause a local reaction, and that it should facilitate or at least not impede tablet making. Except for laboratory experiments, it is also obviously desirable that the excipient itself should ultimately be absorbed. In the case of less water-soluble substances, notably for thyroxine, which in tablet form is absorbed extremely slowly, the excipient should be designed to facilitate rather than to delay absorption. For such a purpose the excipient itself must be absorbed fairly rapidly so that a constant supply of the active substance is shed into the subcutaneous space.

A large number of substances were examined for their possible usefulness as excipients, attention being restricted to those which could be obtained in quantity and which fulfilled some or all of the criteria mentioned above. In rejecting unpromising material it was assumed that a substance which did not behave well when implanted alone was unlikely to improve the characteristics of a compound tablet. This assumption is not necessarily correct, but some limit had to be set to the scope of the investigation. Interesting information, summarized below, was obtained about many of the substances which were rejected for the immediate purpose in view.

Substances rejected

Stearic acid. Two tablets of about 100 mg. were implanted for 20 days. At the end of this time, one appeared to have lost 1 mg., the other was unchanged in weight. In general, stearic acid seemed to behave like cholesterol and to have no advantages over it.

Palmitic acid. Tablets of palmitic acid were found to disintegrate rapidly.

Uric acid, starch, kaolin. Tablets made of these substances all disintegrated or went to a soft paste within 2 days.

Lumisterol, ergosterol, sitosterol. The fact that cholesterol promised to be a useful excipient of the non-absorbed type led to the examination of similar inert steroids in the hope of finding one as effective as cholesterol in retarding the absorption of water-soluble substances, but which ultimately underwent absorption. Lumisterol, ergosterol and sitosterol were, however, examined without revealing any particularly favourable characteristics. A series of 40 mg. tablets of each was implanted into rats for 9 weeks. At the end of this time it was not found possible to demonstrate any regular loss of weight in the tablets, though two of those of lumisterol seemed to have lost a weighable amount. In the circumstances, the substances had no advantage over cholesterol which is more readily available. Moreover, the lumisterol seemed to have

undergone some chemical change, since the tablets, white when implanted, were yellow when removed—a change which has not been observed with any other substance.

Substances of the sulphanilamide group. For the purpose of making tablets of active substances, it is obviously desirable that excipients should be biologically inert. However, the behaviour of substances of the sulphanilamide group when implanted subcutaneously in tablet form, which had been examined in another connexion, suggested that certain of them would make admirable excipients. It seemed, also, that no exception could be taken to an excipient having bactericidal activity; on the contrary, this action might be of considerable value.

Sulphanilamide was found to be absorbed very rapidly after implantation. In rats tablets of 50 mg. completely disappeared in 1 day; tablets of 100 mg. were about three-quarters absorbed in 1 day and almost completely in 2 days. In rabbits absorption was even more rapid, tablets up to 450 mg. in weight being almost completely absorbed in 3 days. Experiment was then extended to the N^4-acetyl, -caproyl, -lauroyl, and -benzyl derivatives. Acetyl sulphanilamide was absorbed rather more slowly than sulphanilamide; tablets of 100 mg. lost only about one-half of their weight in 4 days. The rate of absorption of the other three compounds was of an entirely different order. 100 mg. tablets of caproyl sulphanilamide lost only 4% of their weight in 5 days and 8% in 30 days, while those of lauroyl sulphanilamide lost only about 1% in 66 days. Benzyl sulphanilamide was absorbed at about the same rate as the caproyl compound, but, being more easily obtained, had definite advantages as an excipient. It is dealt with in detail below. Two other compounds of the chemotherapeutic group were examined: sulphapyridine and sulphathiazole. Both were absorbed more slowly than sulphanilamide, but more quickly than benzyl sulphanilamide. 100 mg. tablets of sulphapyridine lost rather less than one-half their weight in 30 days; 100 mg. tablets of sulphathiazole lost rather more than half their weight in 10 days. Unlike the sulphanilamide derivatives, both caused some slight local reaction.

Substances used as excipients

Cholesterol. Cholesterol was the first substance investigated as a possible excipient. It has proved very satisfactory for delaying the absorption of water-soluble substances, though the mechanism whereby it does so is not obvious. Probably the effect depends on decreasing the accessibility of the substances to the body fluids. The chief disadvantage of the substance as an excipient is that after absorption of the active substance the cholesterol matrix remains indefinitely in the subcutaneous tissue. The data concerning the behaviour of tablets of cholesterol alone are given in Table 1.

Table 1. *Absorption from tablets of cholesterol*

Duration of implantation days	Weight of tablet (mg.)		% absorption
	When implanted	When removed	
14	81	81	—
14	72	72	—
77	84	84	—
77	57	56	1·9
77	46	46	—

Tablets were implanted for as long as 11 weeks, but one only showed any loss of weight, and this loss was of the order which it is difficult to be certain about in experiments of this type. A test was then made as to whether the absorption was increased when the cholesterol tablet was of the 'sponge' type left after absorption of a highly soluble substance. A 98 mg. tablet was made of 50% dextrose and 50% cholesterol, and implanted for a month. As shown in the next section, the dextrose is absorbed from a tablet of this kind in about 1 day, but at the end of the month the cholesterol sponge still weighed 49 mg.

Benzyl sulphanilamide. As mentioned above, it seemed that some substance of the sulphanilamide group might make an excellent excipient of the absorbable type. Benzyl sulphanilamide seemed to have a suitable rate of absorption and to be available in quantity. The data for absorption are given in Table 2. The figures for 100 mg.

Table 2. *Absorption from tablets of benzyl sulphanilamide*

No. of tablets	Duration of implantation days	Average weight of tablet (mg.)		% absorption
		When implanted	When removed	
5	5	75	72·8	2·9
5	5	100	97·4	2·6
5	10	75	72·1	3·9
9	10	100	96·4	3·6
5	20	75	67·6	9·9
5	20	100	94·6	5·4
5	30	75	67·1	10·5
7	30	100	91·4	8·6
4	56	100	82·5	17·5

tablets are quite concordant, and show that slightly less than 10% is absorbed in the course of a month. With tablets of 75 mg., the figure for 20 days' implantation is rather discordant, but in general the absorption of the smaller tablets, on a percentage basis, is similar to, though rather greater than, that for the 100 mg. tablets, as would be expected.

Dextrose

A tablet of dextrose inserted into the subcutaneous space is absorbed within a few hours, and it seemed useful, therefore, to carry out a model experiment on retarding the absorption of this highly soluble substance.

With cholesterol

It was found that a tablet consisting of dextrose and not less than 50% cholesterol retained its size and shape indefinitely and could be removed cleanly, at any time after implantation, and the loss of dextrose determined by weighing. Tablets containing less than 50% cholesterol disintegrated rapidly. Experiments were therefore carried out with series of tablets weighing approximately 100 mg., containing 10, 25 and 50% dextrose, and implanted for periods up to 15 days.

The results are given in Table 3. Where 90% of cholesterol is present rather more than one-half of the dextrose is absorbed within 15 days. The absorption, however, is not regular, since 30% is absorbed in the first day. With 25% dextrose, more than 80% is absorbed within 15 days, of which half is taken up in the first day. With 50% dextrose, absorption is much more rapid, 90% being taken up within 1 day and nearly

Table 3. *Absorption of dextrose from tablets of dextrose and cholesterol*

% dextrose	No. of tablets	Duration of implantation days	Average weight of tablet (mg.) When implanted	Average weight of tablet (mg.) When removed	Average dextrose absorbed mg.	Average dextrose absorbed %	Average dextrose absorbed Daily %
10	10	1	100·0	97·0	3·0	30·0	30·0
10	17	3	99·8	96·2	3·6	36·1	3·0
10	17	5	99·0	94·6	4·4	44·4	4·2
10	17	10	99·8	95·1	4·7	47·1	0·5
10	15	15	100·0	94·3	5·7	57·0	2·0
25	5	1	100·0	89·4	10·6	42·4	42·4
25	11	3	97·5	83·5	14·0	57·4	7·5
25	5	5	100·0	82·8	17·2	68·8	5·7
25	10	10	100·0	81·3	18·7	74·8	1·2
25	5	15	100·0	79·2	20·8	83·2	1·7
50	12	½–1	99·2	54·2	45·0	90·7	90·7
50	7	2	98·3	52·6	45·7	93·0	2·3
50	2	3	99·5	50·5	49·0	98·5	2·7

all the rest within another 2 days. At all the concentrations examined, therefore, absorption during the first day is much greater than it is later. This relationship between duration of implantation and amount absorbed is in marked contrast to the linear correlation seen in the case of homogeneous tablets, particularly of steroids. The effect is no doubt due to the dextrose near the surface of the tablet being much more accessible than that more deeply embedded in the unabsorbable cholesterol. Even with the present crude technique, however, appreciable amounts of dextrose are still being absorbed during the second week of implantation from tablets in which the initial concentration of dextrose was 10 or 25%, a condition very different from that seen after implantation of tablets of dextrose without excipient. It remains to be seen whether the technique can be improved sufficiently to allow regular dosage of such a highly soluble substance being administered over a period of time by the implantation technique.

The validity of the method of calculating the amount of dextrose absorbed was checked in the case of nine tablets by chemical estimation of the residual sugar. The data are given in Table 4. In the first two experiments the correspondence between

Table 4. *Calculation of residual sugar in dextrose-cholesterol tablets*

% dextrose	No. of tablets	Average weight of tablets (mg.) When implanted	Average weight of tablets (mg.) When removed	Average residual sugar (mg.) By weight difference	Average residual sugar (mg.) By chemical estimation	Average % sugar absorbed By weight difference	Average % sugar absorbed By chemical estimation
10	5	100	96·6	6·6	5·9	34·0	41·0
25	2	92	76	7·0	6·3	69·4	72·6
50	2	94	50·5	3·5	0·2	92·6	99·5

the two methods of estimating the residual sugar is reasonably good. In the third, the discrepancy on a weight basis is considerable, but even so it is not sufficient to disturb seriously the calculation of the percentage of sugar absorbed. It will be noticed that in all three experiments the amount of residual sugar calculated by weight difference is slightly greater than that given by chemical estimation. The

significance of this fact is not clear. Possibly the chemical extraction of the residual sugar from the cholesterol was not complete; more probably, the final weights of the tablets were slightly augmented by dry matter from the body fluid contained in the tablets when removed.

With benzyl sulphanilamide

It was soon found that benzyl sulphanilamide had little effect in retarding the absorption of dextrose. Thus, five 100 mg. tablets containing 25 % dextrose and 75 % of the excipient decreased in weight to an average of 75·6 mg. after implantation for 1 day, and chemical estimation showed an average of only 0·06 mg. of residual sugar in the tablets. Again, five 100 mg. tablets containing 10 % dextrose lost an average of 11 mg. in 1 day, and the tablets when removed contained only 0·03 mg. of residual sugar. Similar tablets implanted for 3, 5 and 10 days showed a progressive but much slower decrease in weight due to the absorption of the benzyl sulphanilamide. At 20 and 30 days tablets which had originally contained 25 % dextrose and 75 % benzyl sulphanilamide were found to have disintegrated; presumably the benzyl sulphanilamide 'sponge' left after absorption of the sugar was unable to maintain its shape after absorption had proceeded to a certain stage.

Thyroxine

Thyroxine alone

In a preliminary experiment two tablets of 40 mg. and two of 20 mg. failed to show any weighable decrease in size after implantation into rats for periods up to 16 days. Difficulty was, however, experienced in dissecting out the tablets cleanly, and, at best, losses in weight of less than a milligram could not have been recognized with certainty. This difficulty recurred in all experiments with tablets of free thyroxine alone. In a later experiment five tablets of 25 mg. were implanted into each of two rabbits which were killed, respectively, after 82 and 207 days. The tablets when removed were all soft and tightly encapsulated and those from the first rabbit were too badly damaged to weigh. Four undamaged tablets were obtained from the second animal; they appeared to average a fraction of a milligram less in weight than when implanted. A similar experiment was carried out with 50 mg. tablets, the two rabbits being killed at 54 and 207 days respectively. Four undamaged tablets were recovered at 207 days. These appeared to average a fraction of a milligram more than when they were implanted. It is clear, therefore, that under the conditions of the experiment no weighable amount of thyroxine is absorbed from tablets of 25–50 mg., even in the course of many months. Rowlands [1942] has, however, shown by experiments on thyroidectomized rats, that minute amounts of thyroxine, capable of maintaining normal growth, are absorbed from tablets of this kind.

Thyroxine and excipient

Cholesterol was first used as an excipient with thyroxine in the hope of improving the condition of the tablet on removal and making possible more accurate weighings. Two 20 mg. tablets containing 50 % cholesterol were implanted for 11 days, when it was found possible to remove them cleanly. No loss of weight was detected. A long-duration experiment was then put on in which five 25 mg. tablets containing 60 % cholesterol were implanted into each of two rats. The tablets were removed from the

first rat at 42 days, and from the second at 167 days. In both cases the tablets were firm, and it was found possible to dissect them cleanly. No loss of weight could, however, be detected.

In an attempt to expedite the absorption of thyroxine, trials were made with three different excipients which are themselves absorbed rapidly—dextrose, sulphanilamide and sulphathiazole. In no case did the excipient exceed 50%, but even so, of twenty-one tablets, 20–40 mg. in weight, all except two disintegrated in a few days leaving behind an amorphous residue, presumably of thyroxine. The remaining two, containing thyroxine and sulphathiazole, were still solid and of their original shape, but they could not be removed quantitatively.

A further experiment was carried out using benzyl sulphanilamide as an excipient (Table 5). Comparison of the figures with those for benzyl sulphanilamide alone (Table 2) suggests that the presence of thyroxine slows down the absorption of the

Table 5. *Absorption from tablets composed of* 40% *thyroxine and* 60% *benzyl sulphanilamide*

No. of tablets	Duration of implantation days	Average weight of tablet (mg.) When implanted	When removed	Amount absorbed mg.
5	5	50	49·6	0·4
5	10	50	48·6	1·4
4	15	50	48·2	1·8
5	20	50	47·6	2·4

excipient, thereby stultifying calculations based on weight changes. Work on the physiological effects of such tablets will be required before the usefulness of adding soluble excipients to thyroxine can be judged.

Promising results have been obtained by the use of the more soluble sodium salts of thyroxine, but publication of these is temporarily postponed.

Adrenaline

Preliminary experiments on rats showed that the implantation of tablets containing a high percentage of adrenaline was unsatisfactory. Even small tablets (10 or 25 mg.) of the undiluted substance caused a severe local reaction, while larger tablets (100 mg.) containing 50% adrenaline and 50% cholesterol yielded a dose of several milligrams in a few days and proved lethal in most cases. Tablets containing 25 or 15% adrenaline were more promising in an initial test—there was no severe local reaction and the dosage yielded was not apparently excessive. The experiment detailed in Table 6 was then carried out. From the results it may be concluded that tablets containing 15% adrenaline and 85% cholesterol lose between one-quarter and one-third of their hormone content in 28 days. The figures obtained suggest that the absorption from tablets of this kind is fairly regular over a 4-week period. Satisfactory results were also obtained with tablets composed of 25% adrenaline and 75% cholesterol. Absorption was more rapid, both absolutely and on a percentage basis, than from those containing only 15% adrenaline, 7·5 mg., or about one-third of that originally present, being absorbed in 20 days. Moreover, in spite of the fact that the figures for 8 and 14 days are little different there is no conclusive evidence that the rate of absorption

Table 6. *Absorption of adrenaline from tablets of adrenaline and cholesterol*

% adrenaline	No. of tablets	Duration of implantation days	Average weight of tablet (mg.) When implanted	Average weight of tablet (mg.) When removed	Active substance absorbed mg.	Active substance absorbed %
15	3	2–3	100	101	0	—
15	2	14	100	98	2	13·3
15	3	28	100	95·7	4·3	28·7
25	2	2	100	98	2	8
25	1	4	100	98	2	8
25	1	8	100	96	4	16
25	2	14	100	95·5	4·5	18
25	2	20	100	92·5	7·5	30
25	2	30	100	88·0	12·0	48

tends to decrease within the time covered by the observations. It seems that a 100 mg. tablet containing 25 % adrenaline will supply to the animal rather more than 10 mg. of adrenaline in the course of a month. By increasing the number of such tablets the total dosage over the period could be increased indefinitely and it would appear, therefore, that the technique might be convenient where chronic administration of adrenaline is required.

Insulin

The work quoted in the introduction to this paper has shown that tablets containing insulin alone are absorbed quickly, and experiments were therefore carried out on the possibility of delaying absorption by the use of cholesterol as an excipient. The animals were not maintained under constant conditions of nutrition and temperature and no observations were made concerning metabolism, except as to whether or not the treatment was lethal.

In preliminary experiments on rabbits low-zinc insulin was used in addition to crystalline material. The former was used at 5, 10 and 15 %, the latter at 10 and 15 %, with the cholesterol excipient. Tablets were of approximately 100 mg. The implantation of tablets of all three concentrations was lethal in some or all cases, even when the amount of insulin absorbed from the tablet was not detectable by weighing. With 5 % low-zinc insulin, two out of four rabbits died in 24 hr.; with 10 %, the same death-rate was observed; with 15 %, all three rabbits died within 1 day. With 10 % crystalline insulin both rabbits survived, but with 15 %, two died out of three. The maximum absorption of insulin recorded was 3 mg., or 20 % of that originally present, from tablets containing 15 % of low-zinc insulin. As these preliminary experiments on rabbits were not very promising the work was continued by implanting tablets containing crystalline insulin into rats. The hormone was again used with cholesterol, at 10, 15 and 25 %, the tablets being of 100 mg. Most of the rats died within 2 days with typical symptoms of hypoglycaemia. The death-rates in the various groups are shown in Table 7. In another experiment the implantation of one 50 mg. tablet containing 25 % insulin proved fatal within 3 days in three out of four rats.

In the great majority of the rats dying within 2 days of implantation, there was no detectable loss of weight in the tablets recovered at death. It may thus be concluded that the dose of insulin administered by implantation with fatal results was considerably less than 20 units. Doses up to 20 units of crystalline insulin given as a single subcutaneous injection were not lethal. It may, therefore, be concluded that

Table 7. *Proportion of rats dying within 2 days after implantation of a 100-mg. tablet of crystalline insulin and cholesterol*

% insulin	No. and sex of animals	Dying within 2 days	
		No.	%
10	9 males	6	66
15	15 females	1	7
15	13 males	11	85
25	10 males	10	100

the implantation technique is more effective than a single injection in the case of crystalline insulin.

The data given in Table 7 suggest that the rat shows a sex difference in resistance to the effects of insulin, the females being less sensitive. The present material is inadequate to demonstrate the point with certainty, but of the fifteen female rats only two died before the appointed day for killing, while the immediate death-rate among males was very heavy. All the information given in Table 8, concerning

Table 8. *Absorption of insulin from tablets composed of 15% insulin and 85% cholesterol*

No. of tablets	Duration of implantation days	Average weight of tablet (mg.)		Active substance absorbed	
		When implanted	When removed	mg.	%
3	5	100	100	0	—
4	6–7	100	100	0	—
3	14	100	99	1	6·7
2	29	100	97	3	20
1	42	100	98	2	13·3

the loss of weight of cholesterol-insulin tablets after various times of implantation has been derived from the group of female rats. It will be seen that only some 2–3 mg. are absorbed in the course of a month from 100-mg. tablets containing 15% insulin—about 2 units a day—but the evidence so far as it goes suggests that absorption is fairly regular over this period.

The effect of prolonged implantation on the activity of the unabsorbed insulin was tested by the re-implantation of tablets. Two tablets which had been implanted for 14 days in two female rats quickly proved lethal when implanted both together into a male. A similar result was obtained by the combined reimplantation of two tablets which had previously been implanted separately for 7 and 14 days. On the other hand, the re-implantation of the two tablets which had previously been in position for 29 days did not result in the death of the animal. These tablets, however, had not lost further weight when they were removed after 21 days, so the survival of the animal probably indicates growing inaccessibility, rather than inactivation, of the residual insulin.

Chorionic gonadotrophin

Experiments with chorionic gonadotrophin were started in the hope that it would be possible to evolve a method which would give prolonged dosage by a single administration. Two problems were involved: (*a*) could the absorption of the active substance be delayed by the use of a suitable excipient, and (*b*) would the active substance be destroyed by being damped by body fluids for a comparatively long

period? The data given in Table 9 were obtained by using cholesterol as excipient with an extract of urine of pregnancy (UP 24) which contained about 20 i.u./mg. Stable tablets were obtained using cholesterol in proportions of 50, 75 and 90%.

Table 9. *Absorption from tablets containing chorionic gonadotrophin and cholesterol*

% chorionic gonadotrophin	Implanted into	Duration of implantation days	No. of tablets	Average weight of tablet (mg.) When implanted	Average weight of tablet (mg.) When removed	Amount extract absorbed %
10	Rabbit	2	4	20	18	100
10	,,	3	5	100	93·8	62
10	,,	6	14	100	91·0	90
25	,,	2	4	20	15·5	90
50	,,	2	4	20	10·75	92·5
10	Rat	1	2	100	95	50
10	,,	3	2	100	90	100
10	,,	6	2	100	90·5	95

Tablets containing only 25% cholesterol were found to disintegrate. It will be seen that tablets weighing 20 mg. implanted into rabbits, lost all or most of the extract within 2 days, even when 90% cholesterol was present. In view of the comparatively high activity of UP 24, tablets of 100 mg. were made only with the largest proportion of excipient (90%). It will be seen that in both rats and rabbits the 100 mg. tablets lost all or nearly all the extract within 6 days, a large proportion having been absorbed within 3 days and much within 1 day. It did not prove practicable to compare the effectiveness of UP 24 when absorbed gradually from tablets with that when given irregularly by once-daily injection. The dose of UP 24 giving the optimum response in immature female rats is only 0·25 mg., and it was not practicable to administer this amount in tablet form, even with 90% excipient. The rabbits used for the implantations all showed marked ovarian changes, but it was impossible to arrive at quantitative conclusions. The rats used were males.

Examination was made of the residual activity by extracting the tablets after removal and testing the extract on immature female rats. Five 100 mg. tablets containing 10% UP 24 and 90% cholesterol were implanted into a rabbit for 6 days. When removed they averaged slightly under 90 mg., which suggested complete absorption of the chorionic gonadotrophin. The tablets after removal were thoroughly ground with saline, and extract equivalent to one-half of a tablet was injected over 5 days into each of five immature female rats. The resulting ovarian response was such as to indicate that activity equivalent to about 0·5 mg. of UP 24 had been left in each tablet. The weights of the tablets at removal make it unlikely that more than 0·5 mg. of UP 24 remained in each tablet; it may, therefore, be concluded that the active principle itself had not been selectively absorbed from the rest of the extract and that the activity of the residual material had not been impaired by contact with the body fluids for 6 days.

Pituitary gonadotrophins

The first experiments were carried out with an extract of horse pituitary gland (AP 118B) which was made up into tablets containing 90% of cholesterol or benzyl sulphanilamide and 10% of the extract. The tablets were not very satisfactory, as

they tended to crack owing probably to swelling of the extract powder when damped by the body fluids. However, fairly concordant results were obtained (Table 10) and showed that with cholesterol as excipient about one-half of the extract was absorbed within 3 days and all of it in 5 days. With benzyl sulphanilamide as excipient, the absorption seemed to be rather more rapid, though a figure was not obtained for the 5-day stage owing to disintegration of most of the tablets. These experiments suggested that, using cholesterol as excipient, the absorption of 10 mg. of extract could be spread fairly evenly over a 5-day period by the implantation technique. The high activity of AP118B made it an unsuitable extract for investigating whether any increase in effectiveness could thereby be obtained. For this reason, and following the report by Meyer & McShan [1941], that the effectiveness per mg., and the maximum response obtainable, could be greatly increased by very frequent injection of sheep pituitary extracts, an extract of sheep pituitary gland (AP117B) was chosen for further experiments.

Table 10. *Absorption from tablets containing pituitary gonadotrophin and excipient*

Exp. no.	Composition of tablets	No. of tablets	Duration of implantation days	Average weight of tablets (mg.) When implanted	Average weight of tablets (mg.) When removed	Amount of pituitary extract absorbed %	Notes
1	AP118B 10% Cholesterol 90%	5	1	100	96·4	36	Tablets cracked
2	,,	5	3	100	95·0	50	,,
3	,,	5	5	100	89·4	100	One tablet cracked
4	AP118B 10% Benzyl sulphanilamide 90%	5	1	100	95·0	45	Allowance made for absorption of 0·5 mg. excipient
5	,,	5	3	100	91·8	67	Allowance made for absorption of 1·5 mg. excipient
6	,,	5	5	100	—	—	All tablets disintegrated except one
7	AP117B 10% Cholesterol 90%	5	5	100	96·6	34	Tablets in good condition
8	Residues from Exp. 7	5	5	96·6	96·4	2	% calculated in terms of original content
9	AP117B 10% Cholesterol 90%	8	5	49·5	48·9	12	Tablets in good condition
10	AP117B 10% Benzyl sulphanilamide 90%	4	5	98·8	95·5	8	Allowance made for absorption of 2·5 mg. excipient

The results are given in Table 10, from which it will be seen that over a 5-day period the absorption of the sheep pituitary extract is much less than that of the horse pituitary extract. The difference may possibly, but not probably, be associated with the fact that, in order to study ovarian responses, the implantations of AP117B were made into immature female rats. The effects on the ovaries of the amounts

Table 11. Effect of administration by implantation on efficiency of sheep pituitary extract AP117B

No. of rats in group	Route	Total dose per rat mg.	Increase in ovary weight mg.
10	Subcutaneous injection	5	20
10	"	10	32
10	"	25	43
10	"	50	68
5	Absorbed from cholesterol tablet	0·2	19
5	Absorbed from sulphanilamide tablet	0·8	52
4	Absorbed from two cholesterol tablets	1·25	42
5	Absorbed from cholesterol tablet	3·4	54

absorbed from the tablets are shown in Table 11, in comparison with similar increases in ovarian weight produced by the daily subcutaneous injection of the extract in aqueous solution. It will be seen that the effectiveness of the extract is very greatly increased when it is absorbed from a tablet, perhaps as much as twenty times. Conclusive evidence, however, was not obtained of any increase in the maximum response obtainable with sheep pituitary extract. This failure to confirm the work of Meyer & McShan may have been due to the fact that the dose-response curve for AP117B had a substantially higher plateau than is usual with sheep pituitary extracts. The reason for the increased effectiveness of the extract when absorbed from tablets is not certain, but it may well be due, as in the case of the steroid hormones, to the regularity of the supply from such a source as compared with the irregularity arising from daily injection.

SUMMARY

1. Experiments were carried out on the administration of non-steroid substances by the subcutaneous implantation of compressed tablets, made with or without excipient.

2. Cholesterol and benzyl sulphanilamide were found to be the most satisfactory excipients to use for improving the properties of the tablet, or for delaying the absorption of highly soluble substances.

3. A model experiment was carried out with dextrose, the complete absorption of which was delayed for some weeks by incorporating it in 100 mg. tablets containing 90% cholesterol.

4. Thyroxine is not absorbed in weighable amounts from tablets implanted for many months, and no satisfactory excipient has yet been found to expedite absorption.

5. Promising results were obtained with adrenaline. Using cholesterol as excipient, 100 mg. tablets containing 15% adrenaline yield about 5 mg., and those containing 25% adrenaline about 10 mg., of the active substance in a month.

6. The implantation of 100-mg. tablets containing 10, 15 or 25% crystalline insulin, and cholesterol, was usually lethal in male rats. Data obtained on female rats showed that 2–3 mg. of insulin were absorbed in a month from tablets containing 25% insulin.

7. Chorionic gonadotrophin was absorbed rapidly from tablets even when 90% cholesterol was added as excipient. Residual material present in the tablet after 5 days' implantation was still biologically active.

8. Gonadotrophic extract of sheep pituitary gland was much more efficient in immature female rats when administered over 5 days with excipient by the implantation technique than when given by daily injection.

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THE EFFECTS OF OESTRONE ON THE OVARY OF THE MOUSE

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The action of oestrogens on the development and periodic changes of the female accessory sexual organs of the mammals is well known, but it now appears probable that this secretion also influences the growth of the various parts of the ovary itself. A striking coincidence has recently been demonstrated between the positions of high concentrations of oestrogen in the mouse ovary and local areas of mitotic activity in the germinal epithelium, and it is not unlikely that the three nuclear divisions of oogenesis in this animal are induced by oestrogen [Bullough, 1942*b*]. Further, it appears that the growth of the follicles and corpora lutea, as well as the mitotic activity of the interstitial and connective tissue cells, are due to the action of this hormone [Bullough, 1942*c*].

Experiments were therefore planned to test these theories as far as possible. It has already been shown [Bullough, 1942*d*] that abdominal injections of oestrone cause great mitotic activity of the germinal epithelium of the minnow, *Phoxinus laevis* L., and that this results in the production of abnormally large numbers of oogonia. Similar experiments, of which preliminary accounts have already been published [Bullough, 1942*a*], have now been made in the case of the mouse, and the full results are recorded below.

MATERIAL AND METHODS

The mice used in these experiments were of several different strains, but all were normal females of 5 or 6 months old. They were housed in uniformly warm conditions with 12 hr. of light daily, and were given a varied diet which included grain, dog biscuit, greens, and milk with cod-liver oil on bread. The experiments were performed in the summer of 1941.

A variation of the vaginal-smear technique, described by Bullough [1942*b*], was used to distinguish the phases of the oestrous cycle, and in all cases smears were studied twice daily for at least 10 days before the controls were killed or the injection experiments begun. All the mice used had steady and regular oestrous cycles, and as the ovarian cells are known to show most mitoses in the pro-oestrous and oestrous phases, the experiments were performed on mice in the relatively quiescent dioestrous period. This period was found to last for approximately 3 days, and four mice were killed as controls on each of these days. Each series of injections was started at the beginning of the first day of dioestrus, and completed, in most cases, before the end of the second day when the treatment had caused the animal to pass precociously into the oestrous condition as determined by the vaginal smear.

Preliminary experiments were made using various concentrations of oestrone dissolved in water or in sesame oil, and the injections were given either subcutaneously or abdominally. The following technique was finally devised to produce a maximum effect. The oestrone (Menformon, Organon Laboratories) was dissolved in sesame oil, and at each injection 250 i.u. (25 μg.) in 0·25 ml. of oil were given. Injections were repeated at 12 hr. intervals, the last injection being given 12 hr. before death. It was found necessary for the oestrone to come into direct contact with the ovary, and the solution was therefore directed into the dorsal part of the abdominal cavity. From one to six injections were given, the first being in the close vicinity of the left ovary, and the second in the vicinity of the right. The same alternation was observed throughout the series of injections. Finally, 9½ hr. before killing, each mouse received a subcutaneous injection of 0·1 mg. of colchicine in 0·25 ml. of water. This drug arrested in the metaphase all mitoses occurring during the 9½ hr. interval, and greatly facilitated the study of mitotic activity.

The mice were killed by chloroform, and after the throat, thorax and abdomen had been widely opened, they were fixed whole for 2 days in Bouin's solution. The ovaries were then removed, embedded in paraffin wax, and cut in serial sections 7μ thick. The stains used were Ehrlich's haematoxylin and eosin, and the connective tissue was stained in saffron dissolved in absolute alcohol.

OBSERVATIONS

Germinal epithelium

In order to assess the various rates of mitotic activity of the germinal epithelia of control and experimental ovaries, counts were made of all the mitoses present in the germinal epithelium of one ovary from each animal studied, and the results obtained in this way are given in Table 1. In many of the experimental animals it was found on dissection that the oily solution of oestrone had actually bathed the germinal epithelium, but in others it appeared that the thin connective tissue capsule, which surrounds the ovary and separates it from the general body cavity, had prevented most of the solution from reaching the ovary surface. It is probable that this fact was responsible for the widely varying numbers of mitoses counted in the ovaries of the experimental animals.

The lowest mitosis count from the injected mice (mouse no. 273) was considerably greater than the highest from the controls (mouse no. 173), and the highest count of all, 1818 mitoses present after five injections in mouse no. 257, was actually higher than the maximum noted by Bullough [1942*b*] in normal mice after ovulation. Further, it is seen that the mitoses showed little, if any, relation to the various ovarian structures such as large follicles or corpora lutea.* The great majority were present in those parts of the germinal epithelium unrelated to anything other than groups of very small follicles or masses of stroma and interstitial cells. These were the areas of least tension in which, as a consequence, the germinal epithelial cells were usually cubical, or even columnar, in form.

* For the methods of counting the mitoses and of assessing their proximity to the various ovarian structures reference should be made to Bullough [1942*b*].

Table 1. *The numbers of mitoses present in the germinal epithelia of control and experimental ovaries*

No. of mouse	Killed in*	No. of injections	Mitoses: Unrelated	By large follicles	By young corpora lutea	By old corpora lutea	Total
11	D 1	0	7	0	2	0	9
10	D 1	0	6	2	2	0	10
174	D 1	0	5	4	3	0	12
123	D 2	0	4	7	2	1	14
176	D 2	0	3	4	5	2	14
184	D 3	0	4	12	2	1	19
98	D 2	0	1	3	15	6	25
145	D 3	0	3	9	9	5	26
187	D 3	0	6	17	8	4	35
134	D 2	0	7	8	18	7	40
186	D 3	0	10	21	7	5	43
173	D 1	0	21	13	12	3	49
273	D	1	26	8	18	13	65
276	D	1	29	12	22	13	76
274	D	1	49	21	27	10	107
275	D	1	66	25	19	18	128
277	O 1	1	141	39	27	26	233
95	O 1	4	114	69	31	60	274
247	O 2	4	156	74	32	41	303
248	O 2	4	269	44	30	51	394
93	O 2	4	695	179	92	127	1093
254	O 2	4	725	196	156	178	1255
258	O 2	5	128	56	34	43	261
250	O 2	5	197	62	28	35	322
259	O 2	5	249	111	83	145	588
257	O 2	5	938	426	170	284	1818
256	O 2	6	155	60	102	131	448

* Where D = dioestrus; D 1 = first day of dioestrus; D 2 = second day of dioestrus; D 3 = third day of dioestrus; O 1 = early oestrus; O 2 = full oestrus.

Ovarian follicles

The follicles in the control ovaries were all in conditions typical of the dioestrous period [Bullough, 1942c]. There were large numbers of small primary follicles which showed some mitotic activity, and which were clearly undergoing slow growth. A normal proportion of these were becoming atretic. There were also fewer larger follicles in the rapid growth phase. These contained large reservoirs of follicular fluid, and the cells of the membrana granulosa and of the thecae showed great mitotic activity.

In all the ovaries examined from the experimental mice, irrespective of the number of injections of oestrone given, the small primary follicles, which lacked follicular fluid, were apparently entirely normal. Many, as is usual, were undergoing atresia, but the greater number appeared to be growing at the normal slow rate by the steady mitosis of the cells of the membrana granulosa and the theca folliculi.

The larger rapidly-growing follicles were clearly affected by the injected oestrone although, after only one injection, the effects were slight or even absent. On the average there was some reduction in the number of mitoses among the cells of the membrana

granulosa and the theeae, and in two animals (mice nos. 275, 277) there were also signs of necrosis in a few of the cells of the membrana granulosa adjacent to the reservoir of follicular fluid. These effects were always greatest in the largest follicles. After four injections of oestrone the rate of mitosis in the follicle walls was much reduced, and numbers of necrotic cells were usually present. These two effects were most evident in mice nos. 93 and 254 in which masses of necrotic nuclei and cell debris had been shed into the antrum to float in the follicular fluid. They were always least apparent in the discus proligerus.

The remaining animals, receiving five or six injections, showed similar effects. In most instances the growth of the large follicles was much reduced, and in the largest it had often entirely ceased, but there was considerable variation in the responses of different ovaries. Mouse no. 257, whose germinal epithelium showed the greatest reaction, contained many follicles still showing large numbers of mitoses, whilst on the other hand, in mouse no. 258 mitosis had even ceased in the cells of the discus proligerus of the largest follicles and many necrotic nuclei were present in that region.

Corpora lutea

In the ovaries of the dioestrous control animals mitoses were occasionally seen among the luteal cells especially of the newly formed corpora lutea. Apparently similar conditions prevailed in the ovaries of the experimental mice, and it was difficult to ascertain whether any change had taken place in the normal slight mitotic activity.

Stroma and interstitial cells

As in the corpora lutea, mitoses were occasionally observed among these cells both in control and experimental ovaries, but no difference in the rate of this activity was noticeable between the two groups. If any difference did exist, it was obscured by the considerable variation between individuals.

DISCUSSION AND REVIEW

The influence of oestrogens on oogenesis and ovarian growth

The results recorded in this paper are exactly similar to those obtained in the case of the minnow [Bullough, 1942*d*]. In this fish it was shown that, about the time of ovulation, the sudden rapid production of oestrogen coincides with a burst of mitotic activity of the germinal epithelium resulting in the replenishment of the ovary with great numbers of small oogonia. This effect can be reinforced by abdominal injections of oestrone, and it appears certain that an oestrogen is the agent responsible for the divisions of the germinal epithelial cells in the normal animal. The possibility is also suggested that the sudden production of this hormone in the ovaries at the breeding season is the cause of the two final nuclear divisions, meiosis and mitosis, in the fully developed primary oocyte. Similar phenomena have been observed in the starling, *Sturnus vulgaris* L. [Bullough & Gibbs, 1941; Bullough, 1942*e*]. The cells of the germinal epithelium undergo rapid mitosis at about the time of ovulation and, in the course of the following few weeks, a replenishment of the ovary with new oogonia results. Again it appears probable that a sudden high rate of oestrogen secretion at this time is the controlling factor not only of the first division of oogenesis, but also

of the two final divisions of the primary oocyte. In both these annually breeding animals, no divisions of the germ cells take place except in the short periods just before and just after ovulation, and the greater part of the year is taken up by a long process of yolk deposition which is probably under pituitary control.

The mouse, being a polyoestrous mammal, is naturally different in many respects, and as the ovarian cycle is so short, the disentanglement of the endocrine factors involved is not so simple. However, as in the minnow and the starling, the dependence of the nuclear divisions of the germinal epithelial cells on the female sex hormone is now demonstrated, and in the normal mouse these divisions, resulting in the production of new oogonia, take place most actively immediately after ovulation when the released follicular fluid bathes the germinal epithelium [Bullough & Gibbs, 1941; Bullough, 1942*b*]. At this time also the maturation divisions take place in the eggs. It is shown that the effect on the germinal epithelium can be produced experimentally in the dioestrous mouse by bathing the ovary in concentrated solutions of synthetic oestrone. However, oestrogens have other functions to fulfil in the mammalian ovary. This type of ovary contains far greater numbers of non-germinal cells than do the ovaries of the minnow and the starling, and the process of yolk deposition in the egg is almost entirely absent. Follicular growth is substituted for growth of the ova, and follicular growth involves mitosis as does also the early growth of the corpus luteum. It is now reasonably certain that these mitoses, as well as the less frequent mitoses of the interstitial cells and the cells of the ovarian stroma, are induced by the oestrogen present in the follicular fluid of the antrum [Bullough, 1942*c*].

Ovarian cells appear to require high concentrations of oestrogens in their immediate neighbourhood before they are caused to divide, and subcutaneous injections of very large quantities of oestrone, which is taken to the ovary in relatively low concentrations by the blood, have no effect in stimulating mitosis in any of these cells [Bullough, unpublished]. The germinal epithelium must be bathed by the injected oestrone or the extruded follicular fluid before its cells will react fully, and by far the greatest mitotic activity in the wall of the rapidly growing follicle is in the inner layer of the membrana granulosa which is in contact with the follicular fluid. The pools of liquor folliculi inside the ovary are the centres of steep gradients of mitotic activity extending into and dying away in the surrounding cell mass. Abdominal injections of oestrone solution have a stimulating effect only on the outer epithelium covering the ovary, and the deeper cells, including the follicles and corpora lutea, show no increase in their mitotic rate. On the contrary, if the injections are continued, the growth of the follicles slackens and even ceases altogether with the production of large numbers of necrotic cells. This same effect is produced by subcutaneous injections of oestrone [Bullough, unpublished], and has been explained [Moore & Price, 1932] by a theory, for which experimental evidence was afforded by Meyer, Leonard, Hisaw & Martin [1930], that abnormal quantities of sex hormone depress the rate of production of the gonad-stimulating hormone of the anterior pituitary gland. It now seems that this, in turn, reduces the rate of secretion of oestrogen by the ovary so that the concentration of this hormone in the follicular fluid is reduced, and the mitosis of the cells of the follicle wall ceases. An exactly similar effect has been noted [Bullough, 1942*c*] in the full-grown follicle of the normal mouse just prior to ovulation when it is possible that the oestrogen secretion slackens. The depression of follicle growth in the

injected, as well as the normal, mouse may therefore be due to a reduction in the rate of transfer of the oestrogen from the secreting cells into the antrum. It seems that injected oestrone, taken up by the blood stream, is powerless to restart the mitotic activity of the follicle wall as it is not passed into the follicle centre in sufficient concentrations, and cannot in any other way reach the deeper layers of the ovary.

The endocrine control of the ovary

With the above conclusions in mind, it is now possible to give a theory of the interreactions of the various endocrine secretions which induce and control the rhythmic ovarian changes. It is clear from a mass of research [see reviews of Smith, 1939; Fevold, 1939] that the ultimate control of ovarian development is exercised by the anterior pituitary gland, but how many different secretions of this gland are involved is not yet certain. From evidence obtained in close studies of the ovarian cycles of the minnow [Bullough, 1939], the starling [Bullough, 1942*e*], and the mouse [Bullough, 1942*c*], it would appear that there are at least two separate growth phases of the egg or the follicle, and there may therefore be at least two separate factors exerting an influence on the ovary.

In the first two animals, the minnow and the starling, the primary oocytes in the primary growth phase pass through a period of slow growth when no true yolk is laid down. This phase of development may be matched in the mouse by the slow growth of the small primary follicle which lacks follicular fluid. Injections of oestrone have no effect on any of these growth stages, and in the minnow the small oocytes are not even destroyed by repeated injections of the male sex hormone testosterone propionate [Bullough, 1940]. In the minnow and the starling the period of slow growth gives place suddenly to a period of more rapid growth, the secondary growth phase of the primary oocyte, when changes occur in the affinity of the cytoplasm for stain and the yolk is laid down. In the mammal there is a similar sudden change when the follicular fluid appears and the growth of the follicle is greatly accelerated. The rapid enlargement of the egg with the deposition of yolk in the lower vertebrates and the rapid growth of the follicle in the mouse are both stopped by oestrone injections.

It may well be that the factor controlling the primary growth phase of the primary oocyte in fish and birds has been adapted by the mammals to control the early slow growth of the primary follicle, and equally, whatever controls the rapid growth and yolk deposition in the eggs of the lower vertebrates may have been utilized by the mammals to induce the final rapid growth phase of the Graafian follicle. It appears certain that rapid egg growth and yolk deposition are controlled by a secretion of the anterior pituitary gland [see reviews of Smith, 1932, 1939], and probably this is related to that pituitary secretion, termed the follicle-stimulating hormone, which is known to induce rapid follicle growth in the mammals.

In the lower vertebrates the anterior pituitary not only controls ovarian growth, but as the breeding season approaches it stimulates the ovary to produce maximum quantities of an oestrogen which, as already explained, causes the mitotic activity of the germinal epithelium and possibly also the maturation divisions. In the mammals only this second action of the pituitary secretion remains. It seems certain that the main function of the follicle-stimulating hormone of the anterior pituitary is to cause the cells of the follicle wall, probably the theca interna [Mossman, 1937; Corner, 1938], to

secrete a concentrated solution of an oestrogen which is partly deposited in the antrum and partly carried away by the blood to the body. The high concentration of oestrogen in the liquor folliculi then induces the growth of the follicle and, after ovulation, the mitotic activity of the germinal epithelium and of the developing corpus luteum. The production of oestrogen then slackens, and the mitosis of the ovarian cells is greatly reduced. Luteinization takes place in the corpus luteum, and the animal passes into dioestrus when the cycle begins again with the repeated action of the follicle-stimulating hormone of the anterior pituitary.

It is concluded as probable that most, if not all, the nuclear divisions taking place in the ovary, like those in the accessory sexual organs [see review of Allen, Hisaw & Gardner, 1939] and the skin [H. F. Bullough, 1942], are induced by an oestrogen. It is clear that the threshold concentrations of this hormone necessary to cause mitosis in the cells of different organs vary considerably. Relatively low concentrations carried in the blood stream are sufficient to stimulate growth, for instance, in the uterus, but the highest concentrations of oestrogen in follicular fluid coming into actual contact with the cells are necessary to cause active mitosis in the ovary itself.

Androgens and spermatogenesis

The connexion between the oestrogens and the nuclear divisions of oogenesis, which has been described, makes it interesting to consider what effect androgens have on spermatogenesis. The development of the spermatozoa, unlike that of the ova, is almost entirely a process of cell division. In the female vertebrates studied it has been found impossible, for reasons explained, to cause the full development of a mature egg from the germinal epithelium by oestrone injections alone, but in the male, descriptions have been given of striking effects on the nuclear divisions of spermatogenesis following injections of androgens. The minnow testis, after abdominal injections of testosterone propionate, shows great mitotic activity, and within a fortnight spermatozoa are passed into the vas deferens [Spaul & Bullough, unpublished]. The same effect, following similar injections, has even been induced in the male minnow immediately after the spawning season when the testis is exhausted and very small [Bullough, 1942*d*]. In the mammals, identical results have been recorded by Wells & Moore [1936] in the annually breeding ground squirrel, *Citellus tridecemlineatus*. Injections of androsterone or of testis extract cause the precocious development of spermatozoa in young animals, and their unseasonable development in adults. Wells & Gomez [1937] have also induced active spermatogenesis in young and old, normal and hypophysectomized ground squirrels by injections of androsterone and testosterone propionate.

In species like the rat and the mouse, however, different results have been obtained, and Moore [1939] has summarized the evidence of harmful effects on the testis following injections of androgen. This is, perhaps, not unexpected as in these animals spermatogenesis normally continues at a high rate throughout the entire year. Injections of abnormal quantities of androgens may suppress the pituitary function causing the testes to cease the production of their own supply of hormone. The depressing effects on spermatogenesis of injections of androgens thus appear to be exactly comparable to those already described in the case of the Graafian follicle after oestrone injections into the female. However, in the males much appears to

depend on age and on the dose of androgen given. Moore & Price [1932] have shown that sometimes neither injury nor stimulation follows the injections, and Shay, Gershon-Cohen, Paschkis & Fels [1941], using large doses, have hastened spermatogenesis in young rats. Further, Cutuly & Cutuly [1940] have shown that injections of androgens will induce and maintain spermatogenesis in hypophysectomized rats.

It seems therefore justifiable to infer that the endocrine control of spermatogenesis is similar to that of oogenesis in that the male germ cells, and probably also the other testicular cells, are normally induced to divide by the active internal secretion of an androgen.

GENERAL CONCLUSION

The general conclusion is reached that, following the initial stimulus of the anterior pituitary, the gonads control the nuclear divisions of gametogenesis by means of their own internal secretions, and that, at least in the ovary, the divisions of the non-germinal cells are also under the control of these sex hormones. Each hormone is, however, specific in its action, and stimulates only a gonad of its own sex. Oestrogens inhibit testis growth in fish [Bullough, 1940], birds [Ringoen, 1938], and mammals [Golding & Ramirez, 1928], and conversely, androgens produce disruptive changes in the ovary.

SUMMARY

1. Large doses of oestrone were given to adult female mice in dioestrus by abdominal injections into the vicinity of the ovaries. In all cases the mitotic activity of the germinal epithelium was stimulated, and after five injections a maximum of 1818 mitoses was counted in the epithelium of one ovary.

2. The normal rapid growth of the Graafian follicles was reduced, and in some cases it ceased entirely with the production of large numbers of necrotic cells. The largest follicles were affected first, and inside each follicle the discus proligerus resisted the effects of the injections longest. The small primary follicles remained unaffected.

3. No significant changes could be traced in the corpora lutea or in the interstitial and connective tissue cells.

4. A theory is given of the endocrine mechanism controlling the normal vertebrate ovary, and the conclusion is reached that, following an initial stimulation by a secretion of the anterior pituitary gland, the ovary controls the nuclear divisions of oogenesis, as well as the mitoses of the various non-germinal ovarian cells, by means of its own internal secretion of oestrogen. Evidence is also given of a similar control of spermatogenesis by androgen in the testis.

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STUDIES ON THE PARATHYROID OF THE MOUSE

1. THE CYTOLOGY OF THE NORMAL GLAND IN RELATION TO ITS SECRETORY ACTIVITY

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The most important recent investigations of the cytology of the parathyroid gland in mammals are those of Rosof [1934] and de Robertis [1940, 1941], both of whom studied the rat. These investigators described the Golgi apparatus and mitochondria, the former correlating changes in them with the phases of a secretory cycle and the latter describing a reduction of the Golgi material after injection with parathyroid extract and a hypertrophy after the experimental induction of rickets on a low-calcium and low-phosphorus diet. Both workers observed dark osmiophil and light non-osmiophil cells, and de Robertis described a disappearance of the dark cells following parathyroid-extract injection and an increase with low-calcium and low-phosphorus diets.

With a view to carrying out similar experimental studies on the mouse parathyroid the present investigation of the normal cytology was first undertaken. It was also of interest to discover whether the rather complicated secretory cycle described by Rosof was also shown in a closely related animal.

MATERIAL AND METHODS

Glands were studied (principally from male animals) from mice whose ages ranged from 1 to 130 days and over. The animals were fed a balanced diet of cereals and fresh vegetables, with cod-liver oil given in addition once a week.

The animals were killed by decapitation, and the laryngeal region of the trachea, with its associated thyroid and parathyroid glands, was rapidly dissected out and fixed for 24 hr. in Champy's fluid. Certain of the glands, after washing, were impregnated to show the Golgi bodies by the Kolatschev method, being incubated at about 35° C. in 2 % osmic acid for 5 days in the dark. After dehydrating, clearing and imbedding in wax, sections were cut at 3–5μ.

In three instances 0·1 mg. of colchicine in 0·25 ml. of water was injected subcutaneously in adults 9½ hr. before death, and in another four cases young individuals were injected with equivalent doses in proportion to their body weights. In these cases a small portion of the tongue was fixed with the other tissues, the mitotic activity of its germinal epithelium being used as an indicator of the effectiveness of the injection.

A few glands were fixed in Regaud's fluid for purposes of comparison, but this fixative was found to be of very little value for detailed cytological purposes.

The staining techniques used were designed to show the general histological composition of the gland on the one hand, and its more detailed cytological characteristics on the other. The most satisfactory general stain was Mann's methyl-blue-eosin

mixture. The sections were first bleached in chlorine water for about 1 min., washed thoroughly and stained at about 35° C. for several hours or overnight. After drying the slide as far as possible and differentiating in absolute alcohol, the 'dark' cells (osmiophil cells of Rosof) give a distinctly basophil reaction, whereas the nuclei normally stain pink. The best results are obtained in the central parts of the gland since the peripheral portion is nearly always overfixed. As will be shown below, the basophil reaction is not associated with any specific granules like those recorded for the deer [Grafflin, 1940] and monkey [Cowdry & Scott, 1936].

Sections of glands impregnated to show the Golgi bodies were examined either unstained, or lightly stained in erythrosin, or else by the method used so successfully by Severinghaus [1932] and others on the anterior lobe of the rat pituitary. In outline this method consists, first, in staining for about 5 min. in a saturated solution of acid fuchsin in aniline water placed on the slide (during this time it is warmed up to steaming point three times); and secondly, after differentiating in picric-alcohol and mordanting in 1 % phosphomolybdic acid and washing, in staining the sections for several minutes in the acid-violet—methyl-green mixture described by Severinghaus [1932]. When the sections have been rinsed in water and rapidly dehydrated in absolute alcohol, the dark cells should stand out quite sharply from the rest by virtue of their rather darker greyish blue staining. The colour, however, is not dark enough to obscure the Golgi bodies. The nucleoli are stained red, as are the mitochondria.

The best results for mitochondria were obtained by using either the above method or iron haematoxylin, after a preliminary treatment of the sections with weak acidified potassium permanganate solution, followed, after rinsing in water, by a weak solution of potassium meta-bisulphite. Since the mitochondria are often partially osmicated, some form of bleaching, such as the above, was found necessary to ensure the best results. This treatment, however, had the disadvantage of removing the Golgi impregnation. These stains were also found to be the most satisfactory for the study of nuclear structure.

OBSERVATIONS

For the sake of convenience in description, the parathyroids studied were divided into the three following age groups: (1) 1–15 days, (2) 16–40 days, and (3) over 40 days. All were Champy-fixed.

Parathyroid gland in mice 1–15 days old

In this group, up to the age of about 10 days, the gland is only moderately vascular, and its general composition is uniform, showing none of the lobulation characteristic of the more adult condition. The same uniformity characterizes the cellular composition, which at this stage consists of an epithelioid mass of similar-sized cells (Plate 1, fig. 2). Each cell contains a rather large and conspicuous nucleus characterized by the presence of a variable number (usually at least three) of scattered nucleoli (Fig. 1*a*). The cytoplasm is rather sparse and clear, showing no particular staining reaction, and the mitochondria are in the form of granules scattered through the cytoplasm. The Golgi bodies are in the form of a fairly dense peruke-like net capping one end of the nucleus or closely pressed against the side (Fig. 1*c* and Plate 1, fig. 3).

From about 10 days onwards a series of changes occurs both in the general and cellular composition of the gland. The vascularity becomes increased, and this is

associated with an increase in the development of connective tissue strands, resulting at 15 days in a marked tendency towards lobulation. At the same time, although a generally uniform appearance is preserved in each developing lobule, two new types of cell make their first appearance. Both types of cell are at this stage infrequent in their occurrence. The first is rather smaller than the basic or chief cell type, generally, but not always, oval in shape, and is characterized by the darkness of the cytoplasm —a feature independent of, but intensified by, staining. These cells are not detectable in glands fixed in non-osmic fixatives such as Regaud's fluid. Such cells, which will be referred to as dark cells, do not always give the basophil reaction characteristic of the later stages, possibly owing to the fact that the small size of the gland results in some degree of over-fixation even in the middle, with a consequent effect upon the staining. Dark cells were first observed in an 11-day-old specimen; at 15 days they were well defined but not numerous (Fig. 1*d*).

The second type of cell, the clear cell, is somewhat larger than either of the foregoing types. It has a palely staining nucleus and a cytoplasm which has no specific staining reaction and which is clear except for occasional granular aggregates which may in part be of a mitochondrial nature (Fig. 1*e*). At this relatively early stage of development these cells are infrequent.

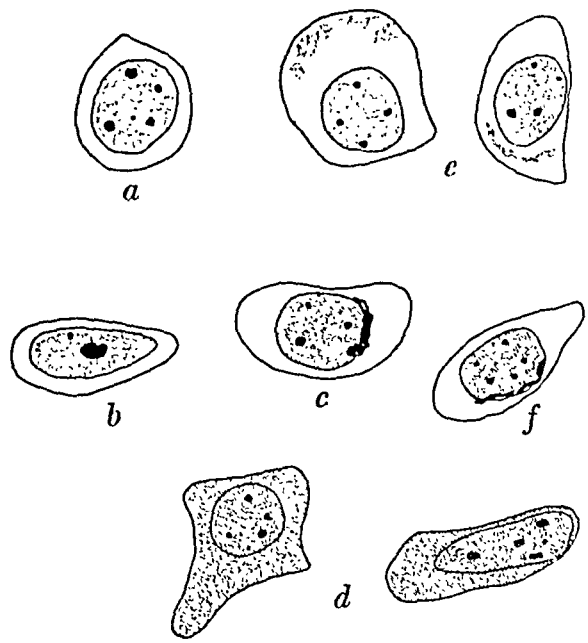


FIG. 1. (*a*) Chief cell from gland of 1-day-old mouse. (*b*) Chief cell from gland of 7-day-old mouse showing uninucleolar nucleus. (*c*) Chief cell from gland of 7-day-old mouse showing peruke-like Golgi body. (*d*) Dark cell from gland of 15-day-old mouse. (*e*) Light cell from gland of 15-day-old mouse. (*f*) Chief cell from gland of adult mouse showing Golgi body. All above figures made from slightly enlarged camera-lucida drawings with 1/12 in. objective and ×6 eyepiece. Champy-fixed and stained with iron haematoxylin (except (c) which was unstained).

It has already been mentioned that the nuclei are of the multinucleolate type, and this holds in general, not only for the chief cell, but also for the dark and clear cells which appear later. Occasionally, however, a type of nucleus such as that illustrated in Fig. 1*b* is seen, and this will be referred to subsequently as the uninucleolar type. Here, instead of a number of scattered nucleoli the nucleus contains one or two very prominent nucleoli, usually centrally placed; there may in addition be a few much smaller ones dispersed about or quite commonly none at all. The often irregular shape of the dominant nucleolus or nucleoli suggests that they may be derived from the fusion of smaller ones. This type of nucleus, although rather infrequently observed in the parathyroids of young mice, becomes increasingly frequent at greater ages and represents the dominant type of the adult. Mitoses were but infrequently observed in the group and then always in the chief cells.

In conclusion it may be stated that in this age group may be traced the early

development of those cell types—the dark and clear cells—which, in greatly increased proportions, are to be found in the adult gland side by side with the chief cells from which they are probably derived. Allowing for individual variations, the latter probably constitute the sole cell type in glands up to 10 days of age at least.

The parathyroid gland in mice 16–40 days old

This age group, although purely arbitrary, shows the transition stages between the juvenile and adult structure. All the parathyroids showed a markedly lobular composition, the cells sometimes being radially arranged around a central blood vessel. The lobulation is associated with an increase in the connective tissue strands, which delimit the lobules, and with a high degree of vascularity.

The cellular composition in general shows a progressive increase in the proportion and definition of the dark cells, with increasing age within the group. These cells began to give a more definite staining reaction with Severinghaus's and Mann's stains. Correlated with these changes was an increase in the size of the gland as a whole, suggesting that there might be considerable mitotic activity. One gland, from a 26-day-old mouse, after colchicine injection, showed only very occasional mitoses in the chief cells, although numerous metaphases were observed in the tongue epithelium. Little weight, however, can be placed on such an observation, since the degree of development within the group as a whole was found to be variable. Further experiments with colchicine injections at different ages are being carried out, which, it is hoped, will throw light on this matter. General histological considerations, however, such as the rapid development of the dark cells, and their frequent occurrence in groups, suggest considerable mitotic activity between the ages of 2 and 5 weeks. There may, for example, be an acceleration of growth and differentiation at the time of weaning, which is normally at about 3 weeks.

In this group also there was an increase in the frequency of clear cells, although they are never, even in the adult, as numerous as the dark cells. Transition types between the dark and clear types could now be made out fairly readily. These cells conform in shape and general appearance to the dark cells, but show varying degrees of lessening in the depth of staining and the cytoplasmic density. Comparison with the adult glands, to be described below, suggests that a secretory cycle is in operation in the glandular cells.

In regard to the two types of nuclei, two specimens from 30- and 36-day-old mice showed a definite increase in the proportion of uninucleolar types. The latter had in fact a composition differing but very little from that of the adult. The remaining glands of lower age within the group showed but occasional definitely uninucleolar nuclei, although there was some evidence of increased disparity in nucleolar size.

Parathyroid gland in mice over 40 days old

Here the characteristics of the preceding group are developed to their fullest extent. The gland is divided up into very vascular lobules separated by connective tissue. All three cell types are present together with transitional forms between the dark and clear cells. The clear cells never constitute a large percentage of the gland, although they may be locally numerous; the dark and transitional cells are predominant, the chief cells being much reduced in frequency. The dark cells, transitional cells and

clear cells may be seen even under low magnification as in Plate 1, fig. 4; the chief cells are only readily made out under high-power magnification. The same figure also shows how the uninucleolate nucleus is the predominating type, but that the original type still exists is shown by Plate 1, fig. 5.

An examination of glands impregnated to show the Golgi bodies (Plate 2, fig. 8) reveals a departure from the uniformity seen in the young gland (cf. Plate 1, fig. 3). The Golgi material ranges in form from strands arranged peruke-like closely around the nucleus, to strands or granules often widely dispersed in the cytoplasm (Fig. 6 *a–d*). The mitochondria appear as short rods when stained with iron haematoxylin; with acid fuchsin staining their shape is not so sharply defined (Plate 2, fig. 7).

A study of preparations impregnated for the Golgi bodies and stained by the Severinghaus method enabled a correlation to be made between the cell types and the Golgi material. The dark cells, often characterized by their oval shape and darkly staining cytoplasm, possess a Golgi body consisting of strands of granules which closely invest part of the nucleus as shown in Fig. 6*a*, Plate 2, fig. 9 and Plate 3, figs. 10 and 11. The nuclei of these cells are, in the majority of instances, of the uninucleolar type, but sometimes they are multinucleolate like the chief cells.

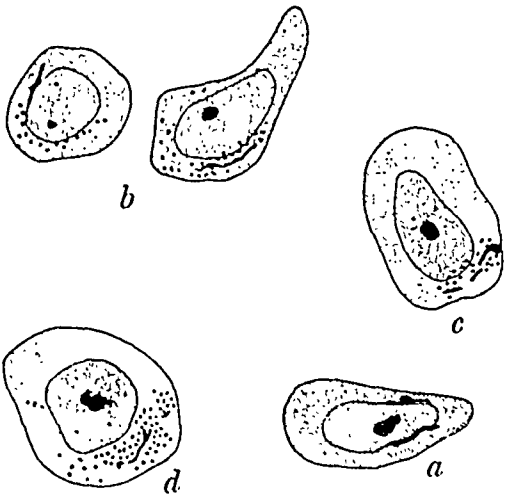


FIG. 6. Cells from the parathyroid gland of adult mice showing mitochondria and Golgi bodies. (*a*) Dark cell with Golgi body close to nucleus. Mitochondria not visible. (*b*) Transition cells with lighter-staining cytoplasm and more widely dispersed Golgi material. (*c*) Later stage of (*b*). (*d*) Clear cell. All above figures made from slightly enlarged camera-lucida drawings with 1/12 in. objective and × 10 eyepiece. Champy fixation and stained with acid fuchsin and methyl-green—acid-violet mixture.

The clear cells are considerably larger than the dark cells, and the cytoplasm, which may be patchily granular, shows no affinity for stains; the Golgi material (Fig. 6*d*, Plate 2, figs. 8 and 9 and Plate 3, fig. 11) is characteristically dispersed at some distance from the nucleus, usually still in the form of threads but sometimes divided into granules. Like the dark cells their nuclei are preponderantly of the uninucleolar type. Some of the cells possess pycnotic nuclei and ill-defined cell boundaries and show every sign of cellular degeneration (Plate 3, fig. 10). As will be seen subsequently, they are regarded as senile secretory cells.

Between these two extreme types are cells which will be referred to as transitional cells (Fig. 6 *b*, *c*, Plate 2, fig. 8 and Plate 3, figs. 10, 11). As the name implies, they are regarded as intermediate forms between the dark and light cells. The evidence for such an interpretation is based on two facts—a series of transitional cells can be constructed showing a progressive, although not great, increase in size, associated with a diminution in the density of staining, and an increase in the diffuseness of the Golgi material. The apparent homogeneity of the cytoplasm of the dark cells is lost with the progression towards the light-cell type. This change is not associated with vacuolation (vacuoles such as those described in the rat by de Robertis [1940] were

never observed) but rather with a progressive irregular cytoplasmic flocculation (Plate 3, fig. 10(2)). At the same time the Golgi strands separate further from the nucleus and tend to break up in the cytoplasm. These changes did not appear to be correlated with any consistent alterations in the staining properties of the nuclei or the distribution of the mitochondria, except that the latter were more widely dispersed in the larger clear cells.

Cells were also present in very small numbers, in which a multinucleolate type of nucleus and cytoplasm of indifferent staining is associated with a Golgi body of the dark-cell type (Fig. 1*f*). Although the Golgi material is not as dense and conspicuous as that in the chief cells of the juvenile gland (cf. Plate 1, fig. 3), its position in combination with the nuclear and cytoplasmic characteristics indicates that these also are chief cells.

DISCUSSION

The working out of a secretory cycle for the cells of a gland is a difficult, if not an impossible, task, unless there are physiological data with which cytological findings can be correlated. Only from such successful correlations can reliable cytological criteria be selected from a mass of observations which are peculiar to the material used or the technique employed, and which are not of general significance [cf. Foster, 1942*b*]. The literature on the experimental cytology of secretion in glands in general and endocrine glands in particular indicates that the behaviour of prosecretion granules where present and of the Golgi bodies is the best guide to cellular activity. Hyperactivity of the pituitary, for example, induced experimentally by the injection of ovarian hormones [Severinghaus, 1936] and synthetic oestrogens [Foster, 1942*a*] is indicated cytologically by hypertrophy of the Golgi bodies and degranulation of the chromophil cells. A similar Golgi hypertrophy has been recorded for the activated thyroid [Ludford & Cramer, 1928], and for the parathyroid glands [de Robertis, 1941]. There is also evidence which suggests that an experimental repression of the activity of a gland may result in some degree of regression of the Golgi apparatus. Castration produces changes which appear to be of this type in the basophil cells of the pituitary [Severinghaus, 1937], and suppression of the activity of the parathyroid gland in the rat by the injection of parathyroid extracts results in regression of the Golgi bodies. If these changes, many of which are reversible, can be regarded as exaggerations of those occurring in a normal secretory rhythm, then such fluctuations might be expected to occur to a smaller extent in the normal gland.

In the mouse parathyroid no prosecretion granules were observed, and the evidence for a secretory cycle is based on an alteration in (1) the staining properties of the cells, and (2) the morphology of the Golgi material. There was found to be a positive correlation between these two sets of observations. Connecting the two extreme cell types, the dark and the clear, was a series of transitional forms, where a progressively lighter staining was associated with a progressively more dispersed Golgi material. Since there is considerable evidence from other glandular cells supporting the view that an enlargement or dispersal of this structure is indicative of secretory activity, it is believed that the transition from dark cells with compact Golgi bodies, to clear cells with dispersed ones, represents a transition from a non-secretory storage phase to one where the secretion has been released. Since the active principle is not in the form of prosecretion granules, it is not possible to associate these changes with a

degranulation process. Although the fact that the progressive decrease in the staining capacity of the cells proceeds in the same direction as the changes in the Golgi material lends support to the above interpretation, such evidence could only be regarded as truly corroborative if it could be proved that the release of a secretory substance is associated with the progressive loss of a stainable material from the cells. Rosof described the dark cells in the rat as being filled with an osmiophil secretory substance, which is ultimately discharged into the blood-vessel after having collected at the secretory pole of the cell on parallel osmiophil strands. According to this investigator these strands are composite derivatives of a Golgi body dispersed into threads and osmicated filamentous mitochondria. Since, after the secretory release, the cell to a large extent loses its osmiophil characteristics, the cycle in the rat appears to consist of a rhythmic synthesis and release of an osmiophil substance. Although the dark cells in the mouse were readily detectable without staining, by reason of their deeper colour, nothing resembling the condition of intense osmication described by Rosof was observed. The cells at the periphery of the glands impregnated by the Nassonov-Kolatschev method often showed definite osmiophil inclusions in the form of granules and threads, but these appearances were undoubtedly due to the over-impregnation which invariably occurs in the outer areas of material thus treated. Although it was found that an osmic-acid fixative was essential to enable the cell types to be differentiated, subsequent bleaching to the extent of removing the Golgi impregnation did not alter the differential staining capacities of the cells. Presumably therefore, the basophil material in the dark cells is an integral part of the cytoplasm, otherwise it would have been removed by the bleaching process, or if in the nature of an uncombined lipoid, would have shown a much more intense osmiophil reaction than was in fact observed.

The next problem that arises is the connexion between the chief cells and the secretory cells. It has been shown that in the cytological differentiation of the gland, the dark and clear cells appear after the chief cells, and since evidence has been presented which indicates that the latter are the final cells of a secretory cycle of which the dark cells are the beginning, then the dark cells must initially arise either as transformations of chief cells or must be derived from them by mitosis. The general difference in shape between these cells suggests that the latter mode of origin is the more likely, and if the chief cells are used up in this process, this may explain their infrequency in the adult gland. Further, it is possible that in the adult gland the dark cells are capable of multiplying themselves, but so far no observations in support of this have been obtained, since mitoses were but rarely observed either in normal or colchicine-treated material. It is hoped that further investigations on colchicine-treated material may throw more light on this problem.

The direct origin of dark from chief cells implies transitional forms, as may also a more indirect origin by mitosis. Such cells might be expected to possess cytoplasmic characteristics intermediate between those of the two cells types (i.e. in regard to staining) and Golgi bodies of the chief cell type. Such cells were not observed, but it is clear that unless they were present in sufficient numbers for all stages to be observed they are liable to be confused with the dark-light transition cells. It seems most likely, however, that the formation of dark cells is a rapid process, perhaps following immediately upon the mitoses of the chief cells and hence not readily detectable.

The final important point concerns the number of secretory phases through which a dark cell passes before reaching a state of permanent exhaustion. In other words, do the clear cells represent a permanent termination of secretory activity, or are they capable of being replenished with the active principle, so that each cell during its active life undergoes a series of phases consisting of an alternate accumulation and release of secretory material? In the anterior lobe of the human pituitary Severinghaus [1936] was able to differentiate between the accumulation and release phases of the chromophil cells by means of a constant alteration in the nuclear staining during one phase as compared with the other. As has been mentioned, no fixed alterations in nuclear staining associated with secretory activity were observed in the parathyroid. Sometimes, however, cells were observed having similar dimensions to the clear cells, but possessing by contrast a basophil cytoplasm and Golgi bodies proximal to the nucleus. These are regarded as transitional cells in the process of either secretion accumulation or secretory release, having passed through at least one previous cycle.

Although little is known of the physiological significance of nucleoli, it is possible that the progressive nucleolar changes associated with the development of the gland from the juvenile to the adult state may have some bearing on what has been said above. If the transition from the multinucleolar to the uninucleolar nucleus occurs as the number of cycles of activity of the glandular cells increases, then this may explain the much greater frequency of the latter type of nucleus in the adult gland. The chief cells being non-secretory would remain multinucleolar in the adult as in the juvenile gland.

Gilmour [1939] and Morgan [1936] have summarized the cell types of the human parathyroid as observed after the ordinary histological fixation processes. During development the first cell type consists of the pale principal cells to which are added in succession the dark principal cells and finally the pale and dark oxyphil cells. Although any comparisons must be extremely tentative the pale and dark principal cells may include types corresponding to the chief, dark, transitional and clear cells of the mouse. Again, the large clear cells of the mouse might be compared with the oxyphil cells of the human and other species, since they both seem to be the result of ageing, but of course these cells have no acidophil reaction in the mouse. It is interesting to note that Waggener [1929] has recorded a rhythmical cytolysis in the parathyroid cells of the anuran, *Rana catesbiana*. Here the cells undergo vacuolization prior to a breakdown which results in the production of a colloidal material; these may have something in common with the apparently senile large clear cells of the mouse.

SUMMARY

1. Up to the age of about 10 days the mouse parathyroid consisted of chief cells only, which were characterized by fairly compact Golgi bodies applied peruke-like to the nucleus and cytoplasm with little affinity for stains.

2. From about 11 days onwards a succession of new cells appeared in addition to the chief cells, but the gland did not acquire a cytological composition comparable with the adult until the age of 4–5 weeks. The new cells were: (*a*) dark cells with a basophil cytoplasm, (*b*) clear cells with a more abundant but practically non-stainable cytoplasm, and (*c*) transitional cells intermediate between (*a*) and (*b*).

3. The variations in form and position of the Golgi bodies and the gradations in the staining capacities of the cells provided evidence for a secretory cycle, in which the dark cells through the transitional stages mentioned above became clear cells. In addition there was evidence to suggest that the cells passed through more than one cycle before becoming senescent.

4. A small proportion of the clear cells possessed ill-defined membranes and showed other signs of cellular degeneration. They were regarded as senescent cells incapable of functioning in further secretory cycles.

5. A progressive change was observed in the nucleolar constitution of the nuclei which ran parallel with the transition from the juvenile to the adult state, and a possible correlation between this observation and the secretory activity of the cells was suggested.

6. In mice between the ages of 1 and 5 weeks the gland underwent a transition from the uniform cell arrangement of the juvenile to the lobular arrangement of the adult.

I would like to thank Professor E. A. Spaul of the Zoology Department, University of Leeds, for his generosity in providing facilities for this work. I wish also to thank Dr J. H. Woodger for his interest and criticism.

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EXPLANATION OF PLATE 1

FIG. 2. Parathyroid of 7-day-old mouse, showing chief cells with their multinucleolate nuclei and scant cytoplasm. Champy fixation and acid fuchsin with methyl-green—acid-violet staining. × 1000.

FIG. 3. Parathyroid of 7-day-old mouse showing dense clumped appearance of the Golgi bodies in the cells. Nassonov-Kolatschev technique. Unstained. × 1500.

FIG. 4. Parathyroid of an adult mouse to show the cellular differentiation. Groups of dark cells are visible along with transitional types and clear cells, the latter having very clear unstained cytoplasm. Champy fixation and acid fuchsin with methyl-green—acid-violet staining. × 280.

FIG. 5. Parathyroid of an adult mouse showing: (1) uninucleolate nucleus of a dark cell and (2) multinucleolate nucleus of a chief cell. Champy fixation. Iron haematoxylin staining. × 1000.

EXPLANATION OF PLATE 2

FIG. 7. Cells of an adult parathyroid arranged around a 'colloid' mass (*c*)—an unusual arrangement. Notice the numerous rod-like mitochondria, most numerous proximal to the 'colloid'. Champy fixation. Iron-haematoxylin staining. × 1500.

FIG. 8. Cells of an adult parathyroid to show the forms of the Golgi apparatus in the cells of an unstained preparation. The preparation was focused primarily to show the Golgi material. (1) A late transitional



FIG. 4



FIG. 5

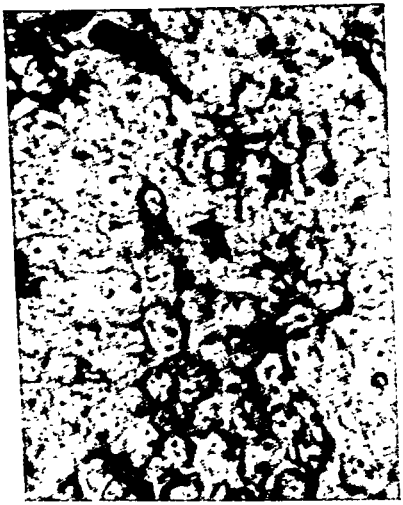


FIG. 2

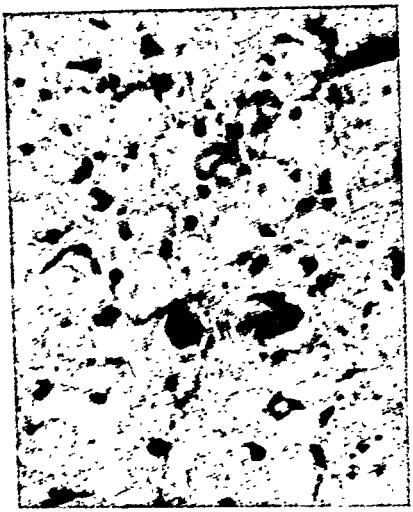


FIG. 3



FIG. 8



FIG. 9



FIG. 7



FIG. 10

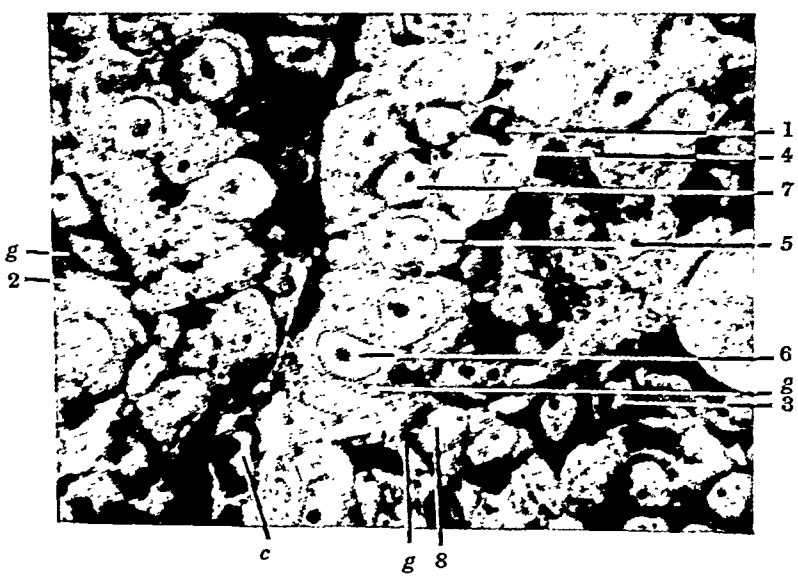


FIG. 11

cell showing Golgi material to right and left of the nucleus. (2) A transitional cell with Golgi material tending to move out into the cytoplasm. (3) A clear cell with a Golgi thread at some distance to left of the nucleus. (4) Another clear cell with Golgi material in the form of a detached mass to the left of the nucleus. *g*, Golgi body. Nassonov-Kolatschev technique. Unstained. ×450.

FIG. 9. Cells of a stained adult parathyroid to show the various types. (1) A dark cell, with dark cytoplasm and a Golgi strand close to the nucleus above and below. (2) A group of dark cells. The Golgi material is rather obscured by the darkness of the cytoplasm. (3) A clear cell, with feebly impregnated Golgi material in threadlike form in the cytoplasm. This cell is probably senescent. Granular mitochondria may be observed in the cytoplasm. Champy-Nassonov-Kolatschev technique; acid fuchsin with methyl-green—acid-violet staining. ×450.

EXPLANATION OF PLATE 3

FIG. 10. Adult gland. (1) Dark cell (possibly at the beginning of transition) showing Golgi body in the form of granules close to the nucleus. (2) Transitional cell with less dense cytoplasm and Golgi material in granules more removed from the nucleus. (3) Senescent clear cell showing Golgi material (*g*2) well out in the cytoplasm. The nuclear membrane is irregular in shape. *g*1, reticular Golgi material of a cell whose nucleus is not in focus. This cell is probably a senescent clear cell. *c*, connective tissue trabeculum.

FIG. 11. Adult gland. (1) Small dark cell with Golgi strand close to and below the nucleus. (2) Elliptical shaped dark cell with Golgi body (*g*) closely applied to the nucleus. (3) Another elliptical shaped dark cell with Golgi strands close to the lower side of the nucleus. (4) Very late transitional or clear cell with light cytoplasm and fragmented Golgi material. (5) This cell possesses the multinucleolate nucleus characteristic of chief cells, but the thread-like Golgi body just detached from the bottom of the nucleus suggests a transitional cell. (6) Clear cell showing part of the dispersed Golgi material in the form of a granule (*g*). (7) Early transitional cell. The cytoplasm is less deeply stained although the Golgi material (at the top and bottom of the nucleus) is still rather close to the nucleus. (8) Transitional cell with Golgi material (*g*) more dispersed in the cytoplasm. *c*, capillary.

Both figures from Champy-fixed Nassonov-Kolatschev preparations. Acid fuchsin with methyl-green—acid-violet staining. ×1500.

COMPARATIVE ACTION OF STILBOESTROL AND OESTRONE ON BODY GROWTH AND ON THE WEIGHT AND GONADOTROPIN CONTENT OF THE HYPOPHYSIS

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The increase in weight of the hypophysis following treatment with oestrogenic hormones has been repeatedly observed [Leonard, Meyer & Hisaw, 1931; Wolfe & Chadwick 1936; Cramer & Horning, 1936; Halpern & D'Amour, 1936; Freudenberger & Clausen 1937; Lauson, Heller & Sevringhaus, 1937; Bacsich & Folley, 1939; Grumbrecht 1940]. Likewise the observation that the gonadotropin content of the pituitary can be reduced by oestrogens is not new [Meyer, Leonard, Hisaw & Martin, 1930]. Recent researches on stilboestrol have shown that this oestrogenic substance also affects the hypophysis in a similar manner [Noble, 1938; Mellish, Baer & Macias, 1940; Page, Russel, Schwabe, Matthews & Emery 1941]. In this paper a comparison between oestrone* and stilboestrol is made using rats for the test animals and the weight and potency of the pituitary as indicative of the effects induced by treatment with the same amount by weight of these two oestrogens.

MATERIAL AND METHODS

The animals were albino rats of the same age and usually litter mates. These were always divided into three groups of nearly equal weight at the time treatment was instigated. All three groups were injected intraperitoneally with equal amounts of sesame oil, which was the vehicle for the oestrone and stilboestrol. A total of 115 donor rats consisting of thirty-six males and seventy-nine females were subdivided into several groups for the purpose of studying effects of the size of the dose, length of treatment, duration of castration, and, to some extent, comparison of sexes. Since these groups are described in the body of the paper their arrangement need not be repeated here.

Stilboestrol readily went into solution in the sesame oil, even in the largest dose used (5 mg. in 1 ml.). The oestrone was also quite soluble in sesame oil, but in the 5 mg./ml. doses it was given in suspension in 50 % aqueous-oil emulsion. This procedure was adopted because the oestrone came in aqueous suspension. The gonadotropic potency of the pituitary was tested by the usual method of pituitary implants in the muscles of virgin rats 25 days of age. The ovaries of the recipients were weighed 5 days after they had received the implants.

* Oestrone in the form of 'Theelin' was obtained through the courtesy of Dr Oliver Kamm, Parke, Davis & Co., Detroit, Michigan, and stilboestrol was obtained through the courtesy of Dr Edward Schwenk, Schering Corporation, Bloomfield, New Jersey.

EXPERIMENTAL

Action of oestrone and stilboestrol on long-castrated rats

Doses equivalent to $\frac{1}{10}$ mg. daily

Thirty-four females, castrated 8 weeks before treatment, were divided into three groups and treated for periods of 10, 20 and 50 days (Table 1). All injections were intraperitoneal and given daily for the first 10 days; thereafter every fifth day. All rats received the equivalent of 0·1 ml. of sesame oil daily. In addition, groups A, D and G received 0·1 mg. of stilboestrol and groups B, E and H 0·1 mg. of oestrone per injection (Table 1).

Table 1 shows the number of rats, kind and duration of treatment, body and pituitary weights of the donors, and the ovarian weights of the recipients. The 10-, 20- and 50-day groups are analysed separately, and in order to increase the number and render a more valid analysis these data are combined into a stilboestrol group (J), an oestrone group (K) and a sesame-oil group (L).

Table 1. *Effect of daily intraperitoneal injections of* 0·1 *mg. of stilboestrol or oestrone on female rats castrated* 8 *weeks previously*

Group	No. of rats	Oestrogen	Duration of treatment days	Body weight Before g.	Body weight After g.	Wt. of hypophysis mg.	Gonadotropin content of hypophysis: Significance ratio*	Gonadotropin content of hypophysis: Wt. of test ovaries mg.	Gonadotropin content of hypophysis: Significance ratio*
A	12	Stilboestrol	10	167	158	11·0	A–B 0·1	39·1	A–B 2·5
B	12	Oestrone	10	171	157	11·1	B–C 0·8	63·5	B–C 0·2
C	12	Control†	10	176	179	11·8	A–C 1·0	67·2	A–C 2·2
D	12	Stilboestrol	20	239	218	13·5	D–E 0·6	18·2	D–E 2·4
E	12	Oestrone	20	244	239	12·7	E–F 0·2	41·9	E–F 2·0
F	12	Control†	20	251	244	12·5	D–F 0·7	66·0	D–F 4·3
G	10	Stilboestrol	50	254	250	13·6	G–H 0·2	24·9	G–H 1·4
H	10	Oestrone	50	237	242	13·9	H–I 1·6	63·6	H–I 1·3
I	10	Control†	50	232	245	12·2	G–I 0·9	44·9	G–I 2·2
J	34	Stilboestrol		218	206	12·6	J–K 0·0	27·5	J–K 3·7
K	34	Oestrone		216	211	12·6	K–L 1·6	55·9	K–L 0·5
L	34	Control†		219	221	12·1	J–L 1·2	60·1	J–L 4·4

* Significance ratio $= \frac{\text{difference of means}}{\text{S.E. of differences}}$. Standard error of differences $= \sqrt{[(\text{S.E.})^2 + (\text{S.E.})^2]}$. S.E. $= \frac{\text{S.D.}}{\sqrt{n}}$.

† Controls were injected with equal volume of sesame oil (0·1 ml. daily).

A reduction in body weight was a constant finding in the stilboestrol-treated rats, occurring in all groups (A, D, G, Table 1) even after brief treatment as shown by a loss of 9 g. at the end of 10 days (group A). These changes were not significant (ratio not shown in Table 1) when the groups (A, D, G) were analysed separately or as a combined group (J, Table 1). The latter group lost 12 g. during treatment, but this mean difference divided by the standard error of the difference (subsequently referred to as the significance ratio) gave a quotient of only 1·7. If this figure was 3 or more the means were considered to be significantly different. The loss of weight in animals treated with stilboestrol was in agreement with published reports [Noble, 1938; Mellish *et al.* 1940; Matthews, Emery & Schwabe, 1941; Deanesly & Parkes, 1941].

The mean body weights of the rats injected with oestrone were also smaller at the end of treatment than at the start in groups B and E, while those in group H, where the duration of treatment was 50 days, were slightly larger. These mean differences were small and not significant when analysed either as single groups or as a combined group (K, Table 1).

The pituitary weight was normal when the treatment was of 10 days' duration, the means being 11·0, 11·1, and 11·8 mg. in the stilboestrol-treated, oestrone-treated, and control rats respectively (Table 1). A small increase in weight of the pituitary was found after treatment with stilboestrol for 20 and 50 days (groups D, G, Table 1), and in the combined group J. All of these increases were slight and not significant; the largest significance ratio was obtained when the data were combined, and here it was only 1·2 (Table 1), indicating that the increase in weight was not significant.

Oestrone also increased the weight of the hypophysis when the treatment was of 20 and 50 days' duration (Table 1), and like stilboestrol the mean differences were small and not significant. Neither was there a significant difference in weight of the hypophysis between the stilboestrol- and oestrone-treated rats. The significance ratios of all these comparisons are given in Table 1, and as shown the largest ratio is only 1·6. Therefore, one must conclude that stilboestrol and oestrone in doses of 0·1 mg. daily did not significantly increase the weight of the pituitary gland in these long-castrated rats.

The gonadotropic potency of the pituitary was reduced in all groups treated with stilboestrol, and the effect was greater than that obtained with oestrone. The mean weights of the test ovaries illustrate this potency. These data and also the significance ratios for each group singly and combined are given in Table 1, which shows that with the exception of group H the pituitary glands of all the stilboestrol- and oestrone-treated groups stimulated the test ovaries considerably less than did the pituitary glands of control rats. The gonadotropin content of the pituitary was significantly reduced by stilboestrol in group D, and in the combined group J where the mean ovarian weights were 18·2 and 27·7 mg. and the significance ratios were 4·3 and 4·4, respectively (Table 1). On the other hand, treatment with oestrone did not yield a single significant change in this pituitary hormone as indicated by the mean ovarian weights of the test rats and the significance ratios which were below 3·0 in every case (Table 1).

In the 50-day experiment the mean ovarian weights of the test rats treated with the pituitaries of control rats were obviously below the expected weight, being only 44·9 mg. (group I) as contrasted with 67·2 and 66·0 mg. in control groups C and F. This variation was eliminated when the groups were combined (group L, Table 1).

Doses of 1 mg. daily

Fifteen long-castrated female rats given 1 mg. of stilboestrol in 0·2 ml. of sesame oil daily for 20 days showed a retardation of body growth. This is indicated in Table 2, where the mean body weights are practically the same at the beginning and end of the experiment. Fifteen similar rats given 1·0 mg. of oestrone in the form of 'Theelin' in 0·2 ml. sesame oil showed a gain of 14 g. in body weight and the controls gained 22 g. (groups M, N, O, Table 2).

The pituitary gland was increased in weight in the stilboestrol- and oestrone-treated groups. The change in the latter group was more than in the former as shown

Table 2. *Effect of daily intraperitoneal injections of* 1·0 *mg. of stilboestrol or oestrone on female rats castrated* 8 *weeks previously. Duration of treatment was* 20 *days*

Group	No. of rats	Oestrogen	Body weight Before g.	Body weight After g.	Wt. of hypophysis mg.	Significance ratio*	Gonadotropin content of hypophysis: Wt. of test ovaries mg.	Gonadotropin content of hypophysis: Significance ratio*
M	15	Stilboestrol	203	206	14·4	M–N 0·7	13·3	M–N 0·9
N	15	Oestrone	187	201	15·3	N–O 3·0	16·0	N–O 5·5
O	15	Control†	200	222	11·9	M–O 2·0	65·2	M–O 5·3

* As in Table 1.

† Controls injected with same volume of sesame oil (0·2 ml. daily).

by comparison with the control group O, and by the significance ratios which were 2·0 and 3·0 in the two groups (Table 2). These changes in pituitary weight although not startling were greater than those shown in Table 1 for smaller doses of stilboestrol and oestrone.

The main purpose in exhibiting Table 2 is to show the drastic reduction in the gonadotropic activity of the hypophysis induced by daily doses of 1·0 mg. of the two oestrogens daily. The ovarian weights of the test rats were equal to or even smaller than the ovaries of untreated 30-day-old rats in our colony (approximately 13 mg.) and sharply in contrast to the ovaries of the recipients receiving pituitary implants from sesame-oil-injected donors. The mean ovary weights were 13·3 mg. for the stilboestrol-treated group, 16·0 mg. for the oestrone-treated group, and 65·2 mg. for the sesame-oil group (Table 2). In spite of the lack of increase in ovary weight, twenty-nine of the thirty test rats in groups M and N had sufficient ovarian stimulation to open the vaginal canal, and six in each group showed positive vaginal smears. This confirms previous reports [Emery, Bash & Lewis, 1931; Emery, 1937; and Heller, Lauson & Sevringhaus, 1938] that the uterus and vagina respond to doses of gonadotropic hormones that are minimal or even subthreshold for increases in ovarian weights. Tests showed that bits of liver, brain and muscle tissue (weighing 15–40 mg.) taken from these donor rats did not bring about oestrus when implanted in castrated rats weighing approximately 70 g. each. Therefore the oestrus found in the test rats of Table 2 must have been due to the pituitary implants acting through the ovaries.

Oestrone and stilboestrol injections in recently castrated rats

A total of thirty-six male rats forming three groups were injected intraperitoneally every 5 days with 0·5 ml. of sesame oil beginning on the day of castration and continuing for 50 days. In addition to the sesame oil, group P (Table 3) received 0·5 mg. of stilboestrol and group Q 0·5 mg. of oestrone at each injection. The results are shown in Table 3.

The body growth of these male rats was little affected by either stilboestrol or oestrone when compared with the growth of those injected with sesame oil. Each group gained approximately 30 g. during the 50 days of treatment.

The mean pituitary weight was also little affected by either stilboestrol or oestrone and the small changes observed were not significant. All three groups showed the expected increase in pituitary weight which follows castration, the mean weights

Table 3. *Effect of intraperitoneal injections of 0·5 mg. of stilboestrol or oestrone every fifth day on castrated male rats. Duration of treatment was 50 days, starting on the day of castration*

Group	No. of rats	Oestrogen	Body weight: Before g.	Body weight: After g.	Wt. of hypophysis mg.	Significance ratio*	Gonadotropin content of hypophysis: Wt. of test ovaries mg.	Gonadotropin content of hypophysis: Significance ratio*
P	12	Stilboestrol	266	297	13·0	P–Q 1·0	27·7	P–Q 0·3
Q	12	Oestrone	260	290	14·4	Q–R 0·6	30·3	Q–R 3·0
R	12	Control†	269	299	13·7	P–R 0·6	65·8	P–R 3·1

* As in Table 1.

† Controls injected with same volume of sesame oil (0·1 ml. daily).

being approximately 14·0 mg. (Table 3), whereas in our colony the pituitary of similar normal male rats weighs approximately 7·5 mg. [Emery, Emery & Schwabe, 1940].

The main purpose of these experiments was to see if the gonadotropic activity of the pituitary would increase after castration even though stilboestrol and oestrone were being absorbed continually from the date of castration. As previously shown the absorption or at least the effect of a single treatment lasts much longer than the 5-day interval between injections employed here [Emery, Matthews & Schwabe, 1941]. Table 3 shows the mean ovarian weights in recipient rats implanted with the pituitary glands of donor rats treated with stilboestrol (group P), oestrone (group Q), or sesame oil only (group R). The results obtained with doses equivalent to 0·1 mg. daily show that although the gonadotropic activity of the pituitary did increase following castration the total activity of each pituitary remained far below that of pituitary glands from castrated male rats treated with sesame oil only. The mean weights of the test ovaries were 27·7, 30·3, and 65·8 mg. in these three groups respectively. The corresponding mean ovarian weight in recipient rats treated with one normal adult male rat pituitary was found to be 17·6 mg., a figure corresponding closely to the mean weight of 17·4 mg. obtained a few years ago [Emery *et al.* 1940].

DISCUSSION

A study of the tables reveals that the depression of body growth was slight, although more evident in rats treated with stilboestrol than in those treated with oestrone. In each group the range of body weights was considerable; this is reflected in the statistical analyses which showed that the mean body weights at the beginning and at the end of the experiment were not significantly different. These rats therefore were obviously not suited for detailed study of the effects of stilboestrol and oestrone on body weight. These data are mentioned here only as confirmatory evidence of the known depressant effects of stilboestrol on body weight and growth [Noble, 1938; Mellish *et al.* 1940; Matthews *et al.* 1941; Deanesly & Parkes, 1941].

The amount of oestrone necessary significantly to reduce the gonadotropic activity of the hypophysis in the long-castrated rat is more than 0·1 mg. (about 330 r.u.) daily as Table 1 shows, but if injections are started on the day of castration this dose seems to be about the minimal amount required to maintain the gonadotropic activity below that of the comparable control (Table 3). This amount of oestrone represents

an increase of five to seven times the amount secreted daily by the normal ovaries, as indicated by an estimated secretion of 200 r.u. at each oestrous cycle in the mouse [Marrian & Parkes, 1930]. Although such a comparison is helpful it is obvious that the injection of hormones can hardly be expected to duplicate exactly the effects produced by the normal ovaries. It would seem that 1 mg. of oestrogen daily is more than is ever excreted by the normal ovaries of rats, for this amount reduced the gonadotropic activity of the hypophysis in long-castrated female rats. Stilboestrol was more effective than oestrone both in reducing gonadotropic activity in long-castrated rats and in retarding the increase that normally follows castration.

SUMMARY

Stilboestrol and oestrone in sesame-oil solution were injected into castrated rats, and their effects on the body weight, pituitary weight and gonadotropic activity of the pituitary gland were compared.

The rats were nearly full grown at the start of the experiment, and thus body growth, although evidently retarded, was not significantly different from that of controls. Growth was reduced more by stilboestrol than by oestrone in the female groups.

The increase in the pituitary weight induced by oestrone seemed to equal that obtained with stilboestrol.

The gonadotropin content of the pituitary of rats castrated 8 weeks previously was lowered considerably by stilboestrol in doses of 0·1 mg. daily, whereas similar treatment with oestrone produced no significant change. When the daily dose was 1·0 mg. both stilboestrol and oestrone completely abolished the gonadotropic activity of the pituitary under the conditions of the test.

The increase in the gonadotropin content of the pituitary following castration still occurred even when stilboestrol or oestrone injections were started on the day of castration and continued to be given in amounts (0·1 mg. daily) that were probably equivalent to five to seven times that normally secreted by the intact ovaries.

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THE REACTION OF THE UTERINE EPITHELIUM OF THE RAT TO OESTROGENIC STIMULATION

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Although an extensive literature exists on the histological changes in the uterine mucosa under the influence of oestrogenic stimulation, no detailed investigation appears to have been made of the behaviour of the Golgi apparatus in the uterine epithelial cells following treatment with oestrogens. It is well known that the internal structure of cells undergoes variation according to their state of functional activity, and the Golgi apparatus in particular has been shown to pass through a sequence of changes in shape and in distribution within the cytoplasm in correlation with various phases of cell activity. Hyperactivity of the thyroid epithelium in exophthalmic goitre and hypertrophy of the prostatic epithelium following administration of androgens, for instance, are each accompanied by characteristic variations in the form and distribution of the Golgi substance.

Experiments were undertaken to determine whether similar precise changes in the Golgi apparatus of the uterine epithelium accompanied oestrogenic stimulation and could be of value as indicators of the degree of stimulation induced by oestrogenic substances.

MATERIAL AND METHODS

Wistar-strain, albino rats, bred in this laboratory and weighing 70–90 g., were selected for these experiments. Sixty-five rats were treated with oestrogens and forty untreated rats were used as controls. Vaginal smears were examined when it was necessary to determine the phase in the oestrous cycle of individual rats before fixation of the uterus. Pro-oestrus and oestrus are accompanied by hyperaemia and distension of the uterus with fluid, while during di-oestrus the uterine wall is collapsed and pale in appearance. These macroscopic changes were sometimes taken as sufficient indication as to whether the untreated rodents were in oestrus or di-oestrus. The effects of the synthetic oestrogens diethylstilboestrol and oestradiol benzoate upon the uterine epithelium were investigated by the tablet implantation technique of Deanesly & Parkes [1937]. Tablets of 5 or 15 mg. were implanted subcutaneously in rats during di-oestrus when the uterine epithelium shows little if any secretory activity. The tablets were removed 8–9 days later and, in order to study the recovery phases of the uterine mucosa, the left horn of each uterus was removed at the same time as the pellet. This uterine horn was fixed immediately after removal to serve as control material for comparison with the remaining right uterine horn taken at varying intervals following removal of the pellet of oestrogen.

The most successful preparations of the Golgi apparatus in the uterine epithelium were obtained by Kolatschev's method as modified by Nassonov [1937]. The sections were counterstained with orange G in clove oil. Aoyama's cadmium chloride

modification of Cajal's silver impregnation technique was also used, but with less satisfactory results. Preparations showing mitochondria were obtained from material prepared by Schridde's and Regaud's methods.

RESULTS

The behaviour of the Golgi apparatus during the normal oestrous cycle

The lumen of each uterine horn in the rat is lined by simple columnar cells, which undergo slight structural changes during the oestrous cycle. A low columnar epithelium is present in the branched tubular uterine glands, similar to that recently described in the mouse by Fekete [1941]. Columnar secretory cells, rod cells and ciliated cells may be distinguished in the uterine epithelium. The secretory cells appear to be merely a functional variation of the rod cells, while the ciliated cells, apart from the cilia, are not different structurally from the simple columnar cells. Similar observations have been made by Aykroyd & Gatenby [1941] on the human endometrium.

Morphological variations occur in the Golgi apparatus of the uterine epithelial cells of the untreated rat during the oestrous cycle. It will be necessary to describe these variations first before any significance can be attached to the more extensive alterations found in the epithelial cells after treatment with oestrogens.

During *di-oestrus* the cells composing the columnar epithelium possess relatively clear cytoplasm. Their nuclei are large and elongated. In the proximal region of the cells close to the basement membrane, the majority of the cells contain fat droplets which stain brown with osmic acid. The droplets disappear shortly after the onset of oestrus. These inclusions should not be confused with the Golgi substance (see Plate 1, fig. 2; Plate 2, fig. 8), which during di-oestrus is condensed near the apical pole of the nucleus, and consists of distorted batons frequently orientated in the cytoplasm transversely to the longitudinal axis of the cell (Fig. 1*b*). The uterine epithelial cells undergo a slight hypertrophy during *pro-oestrus*. The nuclei become more spherical and the apical cytoplasm is slightly distended. Granules and vacuoles are dispersed throughout the cytoplasm. The Golgi substance still remains orientated towards the uterine lumen but it is less condensed and now assumes for the most part a longitudinal polarity within the cytoplasm in close apposition to the nuclear membrane. Fragmentation of the Golgi material is frequently observed (see Plate 1, fig. 3). The most conspicuous cellular changes during the cycle are to be found during late *oestrus* when the epithelial cells become elongated so that the distended apical cytoplasm projects into the uterine lumen, recalling the characteristic bleb cells in the epithelium of the Fallopian tube. The elongated nuclei are confined to the basal region of the cytoplasm and the Golgi substance is dispersed, although restricted to the apical cytoplasm. At this stage the Golgi substance is composed of twisted filaments which are no longer closely applied to the nuclear membrane. In the majority of the cells some of these filaments are intertwined with one another, but their longitudinal polarity is maintained (Fig. 1*a*).

The reaction of the Golgi apparatus to oestrogenic stimulation

The initial reaction of the uterine epithelial cells to oestrogenic stimulation involved a widespread fragmentation of the Golgi apparatus after 16 hr. treatment (Fig. 1*c*). These changes in the Golgi substance were accompanied by hypertrophy of the whole

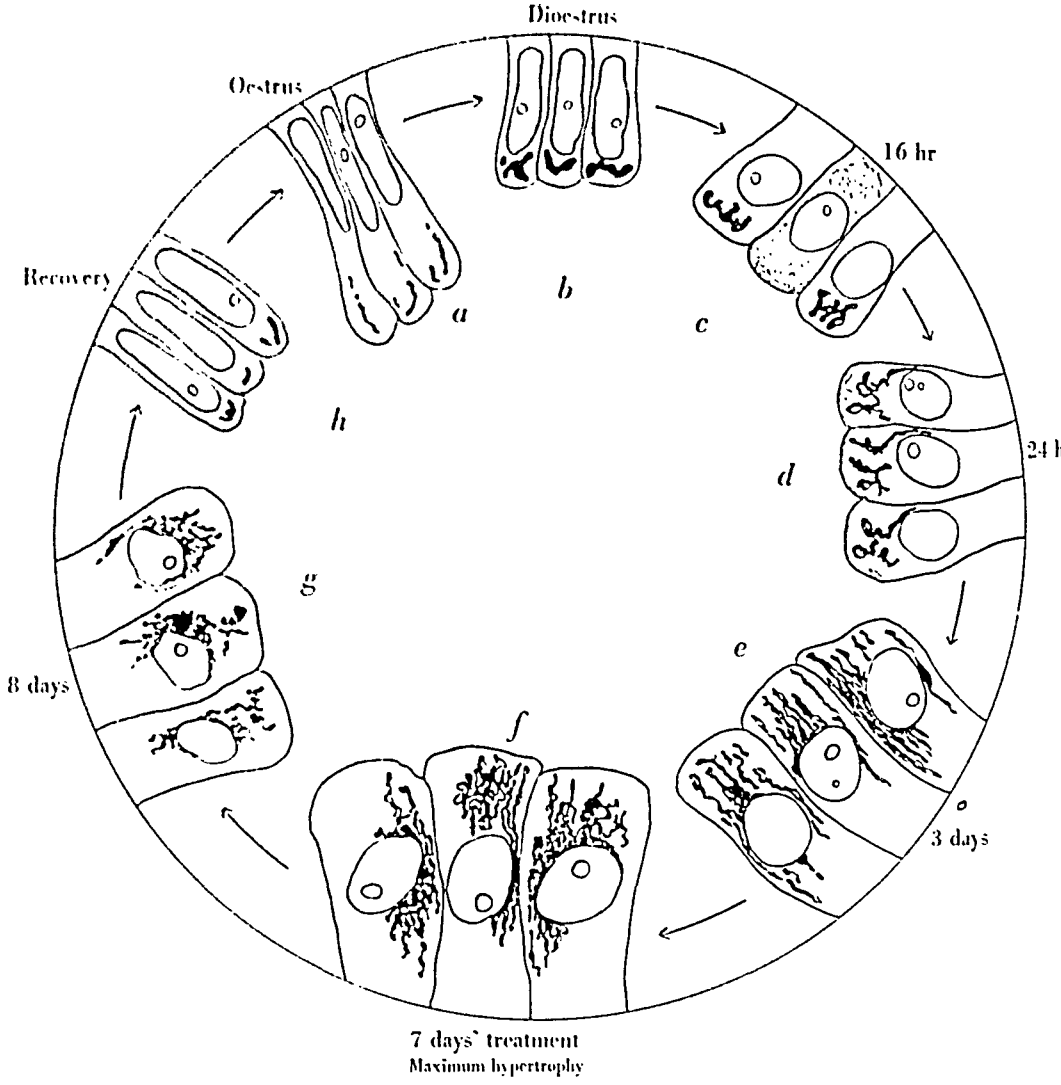


FIG. 1. Camera-lucida drawings of typical epithelial cells, showing the morphological variations of the Golgi apparatus during oestrus and di-oestrus in the normal untreated rat, and its gradual hypertrophy in response to oestrogenic treatment. All drawings were made at the same magnification. (*a*) Elongated cells, typical of the oestrous phase of the cycle, with elongated nuclei confined to the distal ends of the cells. The Golgi material has assumed a thread-like structure, and a polarity longitudinal to the cell axis. (*b*) Cells during di-oestrus. The Golgi apparatus is juxta-nuclear and condensed, often in the form of a distorted baton, lying transversely to the axis of the cell. (*c*) Cells after 16 hr. treatment with oestrogen. The cells have undergone enlargement, the nuclei are spherical and the Golgi substance has hypertrophied. The cytoplasm of those cells containing a completely impregnated apparatus is homogeneous. The cell in the centre has a highly granular cytoplasm, consisting of numerous small osmiophil granules. (*d*) After 24 hr. oestrogenic stimulation. The uterine cells have increased in size. The apical cytoplasm is granular, the hypertrophied Golgi apparatus is very irregular in shape and frequently forms a meshwork. (*e*) Cellular hypertrophy after 3 days' treatment. The Golgi material has increased throughout the cytoplasm. Observe the longitudinal orientation of the Golgi material to the axis of the cell. (*f*) The maximum cellular hypertrophy occurring after 7 days' treatment. The greatly enlarged Golgi apparatus has lost its polarity, having formed an irregular meshwork, frequently extending around the nuclear membrane towards the distal ends of the enlarged cells. (*g*) Cells after 8 days' treatment: the Golgi network begins to fragment, and chromatolysis becomes evident. (*h*) Recovery phase: illustrating the rapid return of the uterine cells and the Golgi apparatus to their normal condition, 7 days after removal of the implanted pellets of oestrogen. The cells, including the Golgi apparatus, although still slightly enlarged, possess a similar structure to those seen during di-oestrus in the normal untreated rat.

endometrium, which attained its maximum enlargement 7 days after insertion of the pellets of oestrogen (Fig. 1*f*). The relative degree of cellular hypertrophy following 6 hr. oestrogenic stimulation can be appreciated best by an examination of Fig. 1*c*. The enlarged epithelial cells were cuboidal and most of the nuclei were spherical. Three distinct cell types could be recognized, all of which were typical of this period of treatment; viz. cells which contained no recognizable Golgi substance with cytoplasm almost entirely masked by small osmiophil granules (Fig. 1*c*), a second type possessing relatively clear cytoplasm with a condensed Golgi apparatus, often forming a close network at the apical pole of the nucleus, and a third or intermediate cell type in which a fragmented polymorphic Golgi apparatus was present together with coarse osmiophil granules dispersed throughout the cytoplasm.

It seems likely from these observations that the Golgi material first responds to oestrogenic stimulation by undergoing fragmentation which is later followed by a recovery phase accompanied by reformation of the Golgi apparatus. Detailed examination of the uterine epithelium showed that the early response of the cells to short periods of treatment with oestrogens did not depend upon the amount of oestrogen implanted. The first obvious changes in the Golgi substance were observed following 16 hr. treatment, similar results being obtained whether the rodents had been implanted with pellets of 5 or 15 mg. of either stilboestrol or oestradiol benzoate.

After 24 hr. the general hypertrophy of the uterine mucosa was more pronounced (Fig. 1*d* and Plate 1, fig. 4). The cytoplasm of the epithelial cells was slightly more granular particularly in the areas bordering on the lumen. The hypertrophied Golgi substance formed a dispersed irregular network which extended in some cases from the surface of the nuclear membrane to the apical margin of the cytoplasm (see Plate 2, fig. 10).

The uterine mucosa at the conclusion of 3 days' treatment had undergone more pronounced morphological changes following oestrogenic stimulation. These changes were well demonstrated in the folds of the surface epithelium, as shown in Fig. 1*e* and Plate 1, fig. 5. The uterine cells were more enlarged than those examined after 24 hr. treatment. A considerable hypertrophy of the Golgi substance had occurred since it now formed loosely drawn out networks made up of intertwining filaments. The irregularly shaped strands forming the meshwork were stretched out longitudinally with reference to the cell axis, often extending from the basal to the apical regions of the enlarged cells, with part of the meshwork closely applied to the spherical nucleus (see Plate 2, fig. 9).

After 7 days, the longitudinal polarity of the Golgi substance, so characteristic after 3 days' treatment, was absent (Fig. 1*f*). Although the uterine epithelium had reached its maximum thickness at this period, no further enlargement of the Golgi apparatus was detected. In fact the network was more condensed (see Plate 2, fig. 11) and frequently occupied a position in the cytoplasm at either pole of the nucleus. The cytoplasm became vacuolated and the distended cells, with apical cytoplasm projecting into the uterine lumen, were a characteristic feature of this phase of treatment.

On the 8th day of treatment the cytological changes were even more pronounced and fragmentation of the Golgi material had begun (Fig. 1*g*). The dispersal of the Golgi substance became more marked in those cells undergoing degeneration and was accompanied by chromatolysis of the nuclei (see Plate 1, fig. 6).

The recovery phases of the uterine epithelium following removal of oestrogen pellets

The rapidity with which the uterine epithelium and the Golgi substance return to the normal unstimulated condition, following removal of the implanted tablets of oestrogen, is strikingly illustrated when one compares Figs. 6 and 7, Plate 1. Fig. 6 shows the enlarged polymorphic Golgi bodies in the hypertrophied epithelial cells following 8 days' treatment with oestrogen. Fig. 7 illustrates the cytological appearance of the uterine epithelium and the Golgi substance from the remainder of the same uterus which was left in situ for 7 days after the pellet had been removed. The uterine mucosa was still slightly enlarged but in general appearance the epithelium was comparable with that of an untreated rat during di-oestrus, except that the Golgi substance was fragmented (Fig. 1 *b*; Plate 1, figs. 2, 7) and tended to accumulate in larger masses in the distal region of the cytoplasm. Aggregations were also seen, less frequently however, around the nuclear membrane. In the apical cytoplasm the Golgi substance was condensed, forming irregular shaped batons, thus resembling the condition of the Golgi substance in the uterine mucosa of the untreated normal rat during di-oestrus.

DISCUSSION

The consistent morphological fluctuations of the Golgi apparatus, which have just been described in the uterine epithelium of the untreated rat during the oestrous cycle, show that normal oestrogenic activity is sufficient to produce cellular changes. It is therefore not surprising that more marked reactions of the Golgi substance follow the experimental administration of oestrogens. The rapid return of the Golgi substance to its normal structure and distribution, when this treatment is terminated, indicates that oestrogenic stimulation does not cause permanent alteration in the structure of the uterine cells.

The preceding survey has further shown that it is possible to correlate variations in form and distribution of the Golgi apparatus with different periods of treatment with oestrogens. The question now arises whether this phenomenon is of value in determining whether substances are possessed of oestrogenic activity, in the same way as the Golgi apparatus of the prostatic epithelium is employed as an indicator of androgenic activity. Although the microscopic examination of vaginal smears is generally regarded as reliable for the estimation of oestrus in animals, the following criticisms have recently been raised against this technique by Hechter, Lev & Soskin [1940]. It has been shown that vaginal cornification is not necessarily a specific test for the presence of oestrogens since yohimbine, a non-oestrogenic alkaloid, is capable of producing rapid vaginal cornification in spayed mice following short periods of treatment. It is also known that several of the carcinogenic hydrocarbons induce similar changes, when injected into rodents [Wright, 1936]. Similarly other investigators have reported extensive keratinization of the vagina as one of the effects of a vitamin-A-deficient diet. The effect in this case is such that the extensive cornification of the vaginal epithelium entirely obscures the normal changes during the oestrous cycle [Wright, 1936]. Freed, Mesirow & Soskin [1937] have also shown that normal cyclic vaginal cornification can take place in rabbits which were found upon laparotomy to possess atrophic ovaries and uteri. Several investigators have, therefore, introduced alternative methods for testing substances for oestrogenic action.

Gillman [1940] has employed fat within the uterine epithelial cells as an indicator of oestrogenic activity. He has described an accumulation of fat which may be stained with fat-soluble dyes like Scharlach R, within the cytoplasm of these cells during the oestrous cycle. This phenomenon has been shown by Aykroyd & Gatenby [1941] to occur during the menstrual cycle in the human endometrium. Gillman [1941] brings forth interesting evidence which shows that an excess of oestrogen in the presence of a low level of progesterone leads to a decrease of fat in the human uterine mucosa.

Astwood [1938] has described a 6 hr. method for the quantitative determination of oestrogens by employing variations in the water content of the uterus. The uteri of 23-day-old rats, following a single injection of 0·1μg. of oestradiol, underwent extremely rapid changes which were accompanied by an increase in the weight of the organ, due entirely to an accumulation of water. The quantitative response of the uterus to oestrogens was calibrated with sufficient precision to permit its use as a method for standardization of oestradiol and oestrone.

It will be unnecessary to comment upon the present status of the Golgi apparatus as a cytoplasmic component since the subject has been reviewed extensively by Kirkman & Severinghaus [1938]. With reference to the present experiments it should be emphasized that the Golgi apparatus is not an artefact arising from the treatment of cells with osmium tetroxide or silver nitrate and that, although little is known of the composition of the substances which make up the Golgi apparatus and still less can be surmised as to its function within the cell, a sequence of changes in the form and distribution of the Golgi substance has been described in almost all exocrine and endocrine glandular cells in relation to states of rest, secretory activity, overstimulation and exhaustion. In the absence of well-defined secretory products in the cytoplasm, the condition of the Golgi substance may be the only means of assessing the state of functional activity of individual glandular cells. According to Cramer & Ludford [1926], Cowdry [1928], Okkels [1931] and Welch & Broders [1940], there is a close correlation between the behaviour of the Golgi substance in the thyroid epithelium and various stages of secretory activity in the thyroid gland in both normal and pathological conditions. In the anterior lobe of the pituitary body Severinghaus [1937] has described phases in the form of the Golgi apparatus typical of rest and stimulation in both the acidophil and basophil cell types. Hyperactivity of the islets of Langerhans induced by prolonged administration of oestrogen in the mouse is accompanied, according to Vazquez-Lopez [1940], by an unusual enlargement of the Golgi apparatus in every cell of the islets. Finally, in the reproductive system Moore, Price & Gallagher [1930] described characteristic cytological responses in the epithelium of the prostate and seminal vesicle to varying concentrations of male hormone. The state of the Golgi apparatus in the epithelium of these glands has proved to be a reliable method of testing preparations for androgenic action.

Whilst it is not suggested that the examination of the Golgi apparatus in uterine epithelial cells should replace the vaginal smear technique as a test for the action of oestrogenic substances, the present experiments indicate that the method is a reliable alternative procedure.

SUMMARY

1. In di-oestrus, the Golgi apparatus of the uterine epithelial cells in the rat forms a relatively compact mass of osmiophil substance at the apical poles of the nuclei.

2. During pro-oestrus and oestrus the cells enlarge and the Golgi material becomes hypertrophied to form a network.

3. Under the influence of oestrogens administered by the subcutaneous implantation of pellets, the uterine cells with their Golgi substance enlarge in direct relation to the period of stimulation.

4. A maximum degree of stimulation involving a dispersal of the Golgi substance throughout the cytoplasm is obtained 7 or 8 days after implantation with oestrogen.

5. Withdrawal of the oestrogenic stimulation is followed by recovery of the uterine epithelial cells and return of the Golgi apparatus to the resting condition characteristic of di-oestrus.

6. Morphological changes in the Golgi apparatus after the administration of oestrogens are similar to those occurring in other types of glandular cells under the influence of an appropriate stimulus, and such changes may be used as reliable indicators of the action of oestrogens.

The author wishes to acknowledge his indebtedness to Dr W. F. Short, Research Department, Boots Pure Drug Co., for the supply of stilboestrol pellets, and also to Mr F. J. Pittock, F.R.P.S., for the photomicrographs.

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EXPLANATION OF PLATE 1

Photomicrographs of the uterine mucosa of untreated and treated rats. All material was fixed and treated by Kolatschev's modification of Nassonov's method, and subsequently stained with orange G in clove oil. Magnifications ×400.

FIG. 2. The uterine epithelium of an untreated rat killed during di-oestrus. The Golgi apparatus is condensed at the apical pole of the nucleus.

Note the dense accumulations of fat droplets proximal to the basement membrane.

FIG. 3. Pro-oestrous phase of the cycle, which is accompanied by a slight enlargement of the uterine epithelium. Pronounced changes in the structure and distribution of the Golgi material have occurred.

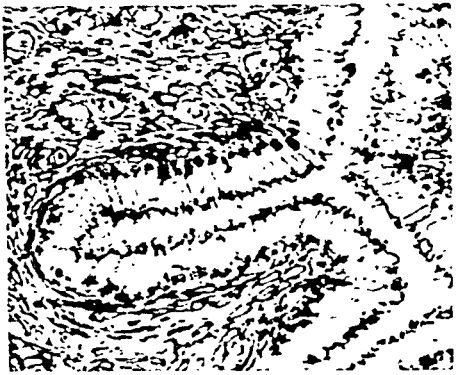


Fig. 2

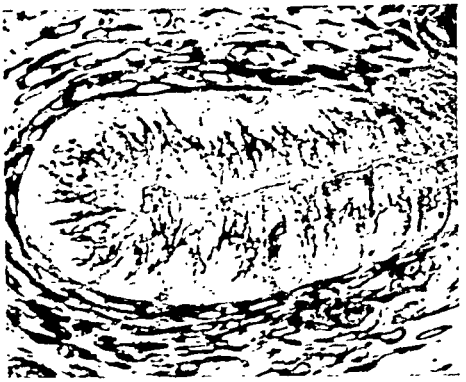


Fig. 5



Fig. 3

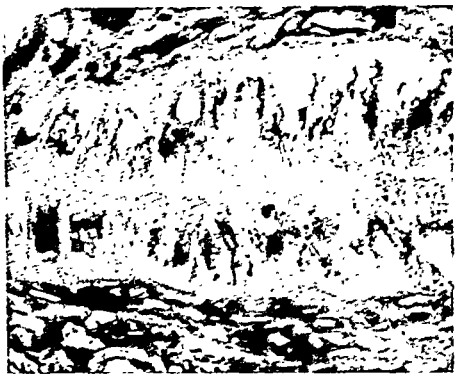


Fig. 6



Fig. 4

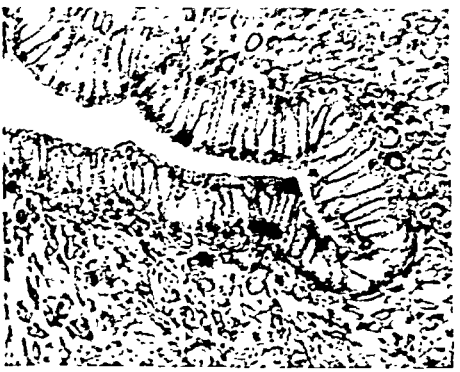


Fig. 7



FIG. 8



FIG. 10



FIG. 9



FIG. 11

FIG. 4. After 24 hr. treatment with oestrogen. The uterine epithelium has enlarged, the Golgi apparatus is hypertrophied, and more irregular in shape.

FIG. 5. Increased hypertrophy of the uterine cells after 3 days' treatment, as shown in a fold of the surface epithelium. The enlarged Golgi apparatus has become more dispersed, and thread-like in structure. In many cells the apparatus has assumed an orientation longitudinal to the cell axis.

FIG. 6. Section of uterus removed at operation after 8 days' treatment, showing maximum enlargement of the epithelium. The orientation of the hypertrophied Golgi material is lost, and fragmentation has occurred. Many of the nuclei are masked by the Golgi substance.

FIG. 7. Section of uterine mucosa from the same rat as that seen in fig. 5, killed 8 days after removal of implanted tablets of oestrogen, showing recovery of the epithelium and Golgi apparatus. Compare with figs. 2 and 3, illustrating the condition in the normal and untreated epithelium.

EXPLANATION OF PLATE 2

Photomicrographs magnified ×4000 of uterine epithelium from normal and oestrogen-treated rats.

FIG. 8. Uterine cells from untreated rat fixed during di-oestrus. The condensed Golgi material is closely applied to the nuclei. Large aggregations of fat are seen in the proximal regions of the cell.

FIG. 9. Typical reaction of uterine cells after 3 days' treatment.

FIG. 10. Enlarged cells after 24 hr. treatment showing the hypertrophied and dispersed apparatus, together with some scattered osmiophil granules.

FIG. 11. Aggregation of hypertrophied Golgi substance, in a greatly enlarged cell after 8 days' treatment.

INDUCTION OF SUPEROVULATION AND SUPERFECUNDATION IN RABBITS

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The discovery of the gonadotrophic hormones prompted the hope that it would readily be possible to induce fertility in immature or anoestrous animals and to augment fertility in those already capable of breeding. Unfortunately, many difficulties have been experienced, owing to the complexity of the endocrine control of the fertile cycle, to the mixture of effects produced by most of the gonadotrophic preparations available, and to the uncertainty of the extent to which the animal's own pituitary becomes involved. Moreover, the augmentation of fertility in breeding animals presents special problems of a mechanical and metabolic nature.

Early in the work with pituitary hormones, Smith & Engle [1927] reported that they had been able to secure the ovulation of twenty to forty-eight follicles in one ovary of the rat or mouse, and later Engle found [1927, 1931] between nineteen and twenty-nine implantations at the 9th–10th day of pregnancy. In a later study Cole [1937] examined the effects of gonadotrophic extracts of pregnant mare's serum on fertility in rats. In mature animals he found that small doses given during metaoestrus increased the average litter size slightly, but no litter exceeded in size the largest found in control animals. Superovulation and superfecundation were found in treated immature animals. Mating and superimplantation followed injection on the 22nd day of life, while 49 % of rats injected on the 26th–31st day mated, and of these, 38 % had more than twenty embryos on the 10th–12th day of pregnancy. The greatest number of living young obtained in one litter was seventeen.

Evans & Simpson [1940] carried out further detailed work on rats. They used a highly purified follicle-stimulating extract of pituitary origin, alone or in combination with chorionic gonadotrophin, and, in keeping with Cole's work, they used rats 26–34 days old at the beginning of the experiment. 10 r.u. of follicle-stimulating extract alone proved to be the optimum dose; addition of chorionic gonadotrophin was of little value. The greatest number of implantations obtained was thirty-four, the average being little more than half this figure; the corresponding figure for the normal colony was ten. The average number of young in litters from treated mothers allowed to go to term was, however, subnormal. These results agree substantially with those obtained by Cole, and Evans & Simpson conclude: 'As the present study will demonstrate, superovulation, that is, the liberation of an increased number of ova, may be followed by the implantation of many of such ova without increase in the average number of young born. Indeed, one may have few or no young born in such cases. The birth of few or no young in pregnancies which are inaugurated with an abnormally high number of successfully implanted and apparently wholly normal embryos is almost surely attributable to defects in uterine function.'

The rabbit offers special facilities for this kind of experiment owing to the fact that ovulation is not spontaneous, and a number of cases of superovulation caused by

administration of gonadotrophic extracts have been recorded incidentally in this species. Pincus [1940] has recently published an account of specific observations. He used prepubertal or pubertal rabbits and investigated the effects of several different preparations of mare's serum, a Fevold follicle-stimulating extract of sheep pituitary, and two horse pituitary extracts. His technique was to give a series of six injections subcutaneously over 3 days to stimulate follicular growth and then an intravenous dose to produce ovulation. He summarizes his results as follows: 'The data indicate that all the gonadotropic preparations will ovulate prepubertal or pubertal rabbits when properly administered, but that mare's serum preparation will induce superovulation only occasionally and then never to the extent of the pituitary extracts; the latter usually causes superovulation in the recipients. The pituitary extracts employed induced, on the average, ovulation of three to more than four times the normal number of ova to be expected from animals of this size. Up to eighty eggs have been obtained from single individuals. The difference between the mare's serum preparations and the pituitary preparations appears to be in the tendency of the former to produce cystic unovulable follicles. Superovulated ova are fertilizable, and mating does not seem to affect the number of eggs produced. Copulation alone cannot substitute for an ovulating injection in young rabbits given a series of priming doses.' Whether all these conclusions would apply to fully mature rabbits is not clear, but as emerges below, that relating to the non-effect of mating does not do so. Pincus obtained up to thirty-six normal embryos in his superovulated rabbits.

MATERIAL AND TECHNIQUE

Rabbits

Most of the rabbits used were drawn from the stocks maintained at the Institute's Field Laboratories. Four distinct breeds were available from this source—Himalayan, Dutch, Copenhagen Albinos and Lop. The first two of these are small breeds, and mature early at a body weight of about 1 kg. [Emmens, 1939], and were therefore unsuitable for work on stimulation of immature rabbits. The Albinos attain a weight of about 2 kg. and the Lop of about 3 kg. before first coming into oestrus. In addition, a number of rabbits, mostly Cross-breds, were obtained from dealers, and were isolated for several weeks before use. All animals were kept singly in separate cages, under which conditions spontaneous ovulation rarely occurs. Even allowing for the variety of breeds used, the material is not as homogeneous as could be wished, since during the later stages of the work the problem of feeding laboratory animals was becoming acute, and the rabbits were on little more than a maintenance diet.

Extracts

In producing the orderly sequence of events necessary to initiate or augment fertility, the two phases of the ovarian cycle, (*a*) follicular maturation, and (*b*) ovulation and luteinization, must be clearly distinguished. Whether the purpose be to initiate fertility in the immature animal or to augment it in adults, the first requirement is to stimulate growth of ovarian follicles. For this purpose extracts having a predominantly follicle-stimulating, rather than a luteinizing, activity are required. Of the various gonadotrophic preparations available at the time these

started, two had this necessary characteristic—extracts of (*a*) pregnant mare's serum, and (*b*) horse pituitary gland [Rowlands & Williams, 1941]. The pituitary extract was selected for a variety of reasons: (*a*) at the time the experiments were started a large stock of well-tested extract was available in the laboratory, (*b*) it seems less violent in its effects on the rabbit ovary than serum extracts, and (*c*) Professor Pincus had found similar extracts prepared at the National Institute for Medical Research to be very effective. A disadvantage in using the pituitary extract was the absence of any International Standard suitable for its standardization. The two extracts used (AP61B and AP118B) were about equally active. A dose/response curve for the effect of AP61B on the ovary of the immature rat has been given by Rowlands & Williams [1941]. Five daily doses of 0·5 mg. increased ovarian size to 60 mg., six times the control size. These pituitary extracts were administered by a series of subcutaneous injections for the purpose of stimulating the follicles ('priming').

To initiate the second phase of the cycle, ovulation and the development of the corpus luteum, a single intravenous injection was given. In a few early experiments AP61B was also used for this purpose, but otherwise two gonadotrophic extracts of urine of pregnancy (*UP10* and *PU11*) were used. These both had an activity similar to that of the International Standard, 10 i.u./mg., and dosage is indicated throughout the paper in i.u.

OBSERVATIONS

Most of the animals were killed in order to make the desired observations on the ovaries and uterus. Where a series of observations was required, however, laparotomies were performed with aseptic technique under anaesthesia. Records were kept of the general appearance of the ovaries and uterus, and of the number of (*a*) mature follicles, (*b*) cystic follicles, (*c*) blood follicles, (*d*) ovulation points, (*e*) corpora lutea, (*f*) normal embryos, and (*g*) atrophic embryos.

Stimulation of follicular development in adult rabbits ('priming')

In the course of the work many observations were made on the state of the ovaries of adult rabbits after a series of five daily injections of the horse pituitary extracts.

In an initial experiment an albino doe was given five daily doses of 1 mg. of AP61B. At the start of injection the ovaries contained a total of six mature follicles; at the end thirteen were present. Several observations were then made, after a similar length of treatment, on animals which were meant for superfecundation experiments, but which failed to mate after priming. Six of these, three Dutch and three Cross-bred, received 5 × 1 mg. of AP118B. All possessed good mature follicles at the end of treatment, but only one, which had thirty-eight follicles, was strongly stimulated. Six rabbits, four Dutch and two Lops, were given 5 × 5 mg. The Dutch responded badly. One showed numerous normal mature follicles at the end of treatment, and another had a few mature follicles only; the remaining two had a large number of cystic and haemorrhagic follicles—clear evidence of over-stimulation. The two Lops responded better, one having a very large number of apparently normal mature follicles.

Optimum duration of treatment

Experiments were carried out on the optimum duration of the priming process. The first point investigated was whether ten daily injections were more effective than

five. The results (Table 1) showed conclusively that the longer treatment led not merely to no increase, but actually to a decrease in ovarian stimulation. This decrease was evident not merely by the lack of or decrease in number of mature follicles at 10 days as compared with 5 days, but also by the general condition of the ovaries which in all except QT93, receiving 10×5 mg., were anaemic and inactive looking.

Table 1. *Relative effectiveness of five and ten daily injections of horse pituitary extract for priming adult females (Albino rabbits)*

No. of rabbit	Daily dose mg.	Number of mature follicles		
		Initial	After 5 days	After 10 days
QT87	1	0	11	0
QT88	1	6	17	0
QT84	1	—	—	0
QT93	2	10	35	27
QT94	5	8	41	10

This result was highly reminiscent of the insensitivity known to follow prolonged treatment with gonadotrophic extracts of heterologous origin. This insensitivity is known to be due to the production of anti-substances by the chronically injected animal, and it therefore seemed surprising that such an effect could appear in 10 days. However, Hamburger [1938] has shown that injection of mare's serum extracts evokes the formation of anti-substances very rapidly in the rabbit, and it seemed possible that horse pituitary extracts might cause a similar rapid response. An experiment was therefore designed to see whether the insensitivity beginning to appear after ten daily injections could be overcome by drastic increase of dosage. Details are given in Table 2. Owing to the rapid succession of laparotomies involved, only one ovary

Table 2. *Onset of insensitivity to horse pituitary extract*

Days of injection	Daily dosage mg.	Ovary examined	State of ovaries at end of period	
			QT89	QT90
1–10	2	Right	Some medium follicles. None mature	Large number of cystic follicles and blood follicles. Some medium follicles
11–15	4	Left	3 medium follicles and a few small	Ovary smaller. Some small blood follicles. 4 medium follicles
16–22	8	Right	1 medium follicle only. No corpora lutea or blood follicles	Ovary small. One medium follicle. Several small blood follicles, obviously remains of blood follicles and cystic follicles seen on 10th day
22–27	16	Both	Ovaries small and immature-looking. No large or medium follicles	Both ovaries small, 3–4 medium follicles in each

was examined at a time (prior to killing) and no initial examination was made. The results show conclusively that even a geometric rise in dosage every few days fails to overcome the insensitivity, the increase in which apparently keeps pace with the rising dosage. It is evident that no further effect is produced with horse pituitary

extract after the tenth day, and that even greatly increased dosage does not prevent the ovary of the continuously injected animal from sinking into a quiescent condition.

Two further experiments were performed with a view to obtaining quantitative information. In the first experiment, eight rabbits were given 2 mg. of AP118B daily. Two of the animals were killed after 5 days, two after 10 days, two after 15 days, and two after 20 days. The ovaries were weighed after being fixed. The largest ovaries were obtained after 10 days' injection; the ovaries were smaller after 20 days' injection than after 5 days'. The number of ripe follicles was also greatest after 10 days' injection. In the second experiment a similar series of rabbits was injected, the dosage, which started at 2 mg. daily, being doubled each 5 days. The weight of the ovaries was greatest after the second 5-day period, during which the dosage had been 4 mg. daily. At the end of 20 days, during the last 5 of which the daily dosage had been 16 mg., the ovaries had reverted to the control size. The number of ripe follicles was again greatest after 10 days of treatment. In these two experiments there were large numbers of cystic and haemorrhagic follicles after ten daily injections. To produce a large crop of normal follicles preliminary to superovulation, therefore, there is nothing to be gained by prolonging the injections of horse pituitary extract beyond 5 days.

A comparison was then made as to whether a 3-day period of priming could be made as effective as the 5-day period. The details are given in Table 3, which shows

Table 3. *Relative effectiveness of three and five daily doses of horse pituitary extract for priming adult females*

No. of rabbit	Breed	Daily dose mg.	Number of mature follicles present		
			Initial	After 3 days	After 5 days
QT289	Lop	1·0	3	6	11
QT290	Lop	1·0	3	6	7
QT291	Lop	1·0	0	9	9
QT284	Lop	2·0	4	9	17
QT285	Albino	2·0	7	7	12
QT286	Lop	2·0	6	15	31
QT287	Lop	2·0	5	12	17
QT279	Lop	5·0	6	9	11
QT280	Lop	5·0	3	7	21
QT281	Lop	5·0	0	13	9
QT282	Albino	5·0	5	3	8
QT283	Lop	5·0	1	14	12

that 1 or 2 mg. daily produced a better crop of ripe follicles after 5 days than after 3 days, and that 5 mg. daily gave a less favourable result than did 2 mg. daily. The fewer number of normal mature follicles produced by the higher dosage was correlated with the presence in the ovaries of large numbers of cystic and haemorrhagic follicles, and it was evident that 5 mg. daily led to gross over-stimulation of the ovary and was too high a dosage for priming. With 2 mg. daily only a few cystic and blood follicles were found after 5 days. With 1 mg. daily these abnormal structures were rare. It may be concluded, therefore, from the whole of the work dealing with the duration of the priming process, that 5 days of treatment with horse pituitary extract is optimal, the best dosage being 2 mg. daily for Lop and Albino rabbits, and 1 mg. for Dutch or Himalayan rabbits.

Induction of ovulation by mating of the primed female

In considering the question of inducing superovulation from the primed ovary, the first question arising is whether the ovulation-producing act of mating is sufficient to cause ovulation in a greater than normal number of follicles, i.e. whether it causes the liberation from the anterior pituitary body of amounts of ovulation-producing substance adequate to produce superovulation. The work of Brambell & Parkes [1932] and of Hill [1934] shows that the amount produced in the normal mated rabbit is greatly in excess of that required to cause ovulation of the normal number of follicles, and might well, therefore, be adequate to cause ovulation of a supernormal number. In practice excellent results (Table 4) were obtained with rabbits, Lop, Dutch and

Table 4. *Induction of ovulation by mating of primed females*

Daily priming dose mg.	No. of rabbits			Total follicles ovulating
	Primed	Mating	Ovulating	
1	11	4	3	27
2	10	9	8	187
5	11	5	1	26

Cross-bred, primed by five daily injections of 2 mg. of AP 61 B or AP 118 B. Nine out of ten animals mated and eight of them ovulated, the average number of ruptured follicles being twenty-four. Of eleven rabbits primed with five daily doses of 1 mg. only four accepted the buck. This low percentage may perhaps be attributed to the fact that these particular experiments were carried out in autumn when even laboratory rabbits are less reproductively active, and when the priming dose may, therefore, be higher. This explanation is supported by the fact that 2 mg. daily usually seems to be an excessive priming dose for Dutch rabbits, whereas in this set of experiments it caused, as mentioned above, a very favourable response in the ovary. The 5 mg. priming dose, however, caused, as usual, gross over-stimulation of the ovaries, and the resulting large crop of cystic and haemorrhagic follicles was associated with a very low percentage of mating, and ovulation in only one of the five which took the buck.

Induction of ovulation by intravenous injection into the primed female

The next stage of the investigation concerned the necessary or optimum dose of chorionic gonadotrophin for causing superovulation from the prepared ovary. Dutch and Cross-bred rabbits were used, primed with five daily doses of up to 5 mg. of horse pituitary extract. Results are shown in Table 5, and the following conclusions may be drawn.

(*a*) In unprimed Dutch rabbits a dose of 10 i.u. of chorionic gonadotrophin was required to cause ovulation of the full number of follicles. Five units produced ovulation of only about one-half the number, while increasing the dose above 10 units did not result in the ovulation of a supernormal number of follicles.

(*b*) Where an effective priming was carried out ($5 \times 1{\cdot}0$ mg. of pituitary extract) a dose of 5 units of chorionic gonadotrophin caused ovulation in many more follicles than in the unprimed rabbit. Ten units gave the maximum response obtained and further increase in dose did not increase the effect.

Table 5. *Induction of ovulation in primed females by intravenous injection of chorionic gonadotrophin*

Breed	Daily priming dose mg.	Dose of chorionic gonadotrophin i.u.	No. of rabbits	Number ovulating	No. of follicles	
					Total	Average
Dutch	Not primed	5	4	4	13	3·25
		10	5	5	33	6·6
		20	4	4	29	7·25
		50	5	5	34	6·8
	1·0	5	5	5	75	15·0
		10	4	4	87	21·75
		20	5	5	104	20·8
		50	4	4	81	20·25
	2·0	5	5	2	24	4·8
		10	4	3	60	15·0
		20	4	3	40	10·0
Cross	2·0	10	4	4	87	21·75
		20	4	4	132	33·0
	5·0	20	2	2	53	26·5
		10	5	1	1	—
		20	5	3	4	—
		50	5	0	0	—

(*c*) In Dutch rabbits priming with 5 × 2·0 mg. led to a marked decrease in the number of ovulations obtainable. This decrease was associated with the appearance of masses of haemorrhagic and cystic follicles in the ovaries of several of the animals.

(*d*) With the Cross-bred rabbits 5 × 2·0 mg. of AP118B was a very favourable priming dose, and it seemed that more than 10 units of chorionic gonadotrophin were required to obtain the maximum ovulation from the large number of mature follicles produced.

(*e*) Where the priming dose to the Cross-breds was raised to 5·0 mg. very few ovulations were obtained. The ovaries were grossly over-stimulated and consisted mainly of cystic and haemorrhagic follicles.

As a general conclusion from this series of experiments, it may be said that optimum priming was achieved by five daily injections of 1 mg. of AP118B (Dutch rabbits), of 2 mg. of AP118B (Cross-breds), and that maximum ovulation was brought about by the intravenous injection of a single dose of 20 units of chorionic gonadotrophin.

Induction of ovulation by mating and intravenous injection

A few records were obtained from rabbits which were both mated and injected intravenously with chorionic gonadotrophin at the end of the priming period and examined soon afterwards. Two large ovulations (46 and 47 ruptured follicles) were obtained in this series, but otherwise there was nothing to suggest that the dual stimulation resulted in any outstanding response. The results of the superfecundation experiments described in the next section, however, make it likely that a lower dose of chorionic gonadotrophin is required when mating takes place.

Induction of superfecundation in primed rabbits

It has already been reported by Padootcheva, Vunder & Zawadowsky [1935] and shown conclusively by Pincus that the eggs produced as the result of artificial stimulation of the ovary are perfectly normal as regards their maturation changes and their susceptibility to fertilization and development. Confirmatory results were obtained in an experiment carried out for a different purpose in which up to fifty-four normally segmenting eggs were recovered from the fallopian tubes of rabbits receiving five daily doses of 2 mg. of AP118B and 50 units of chorionic gonadotrophin intravenously before artificial insemination. Experiments dealing with the subsequent history of such large numbers of fertilized eggs are recorded in Table 6, which shows the

Table 6. *Superfecundity in rabbits primed with horse pituitary extract, mated, and injected intravenously with chorionic gonadotrophin*

No. of rabbit	Breed	Daily priming dose mg.	Dose of c.g. (i.u.)	Time killed or laparotomized after mating days	No. of implantations	Notes
QT83	Albino	1	None	8	24	—
QT85	Lop	1	None	17	2	Preparatory treatment continued for 10 days
QT104	Himalayan	1	10	8	34	—
QT109	Lop	1	10	16	10	7 embryos being reabsorbed
QT110	Lop	1	10	8	5	Only about 12 corpora lutea
QT143	Cross	1	10	11	21	Several abnormal embryos
QT145	Cross	1	10	11	16	—
QT146	Cross	1	10	11	13	—
QT148	Cross	1	10	11	22	—
QT149	Cross	1	10	11	9	—
QT86	Lop	2	None	8	16	—
QT108	Dutch	2	5	9	22	21–24 corpora lutea
QT107	Himalayan	2	5	16	20	All being reabsorbed
QT221	Cross	2	10	8	33	—
QT95	Albino	2	10	9	12	Very large no. of corpora lutea
QT97	Lop	2	10	9	38	Very large no. of corpora lutea
QT98	Lop	2	10	9	16	—
QT105	Dutch	2	10	9	16	About 23 corpora lutea
QT106	Himalayan	2	10	12	12	Several embryos being reabsorbed
QT96	Albino	5	10	9	21	Very large no. of corpora lutea

number of implantations found at various days after mating in primed rabbits. All the rabbits except three received an intravenous injection in addition to mating. Albino, Lop, Himalayan, Dutch and Cross-bred rabbits are included. No adequate data are available for the normal size of litter in these different breeds, but the usual litter size in the Dutch and Himalayan may be considered as being four to six, and in the Albino and Lop as six to nine. It will be seen from Table 6 that where mating alone was relied upon to cause ovulation, implantations of sixteen and twenty-four embryos were found at 8 days. The other animal in this group, QT85, is not strictly comparable, since the preparatory treatment was continued for 10 days, when, as shown above, the priming effect is beginning to wear off, while observation was not made until 17 days after mating, when, as shown below, the number of implantations is decreasing. Most of the rabbits receiving an intravenous injection at the time of

mating implanted a quite abnormal number of embryos, thirty-eight being the largest number recorded. In several of these rabbits very numerous corpora lutea were present, and it is impossible to give any accurate figure for the difference between the number of corpora lutea and the number of embryos. The difficulty of giving a figure for the number of eggs which failed to get fertilized, or which were reabsorbed earlier than 8 days, is increased by the fact that many of the corpora lutea may have been derived from follicles which luteinized without ovulation. Observations on the subsequent history of the embryos at the end of the first third of pregnancy are entirely in accordance with the report by Evans & Simpson [1940] that the number of living young produced by superfecundated rats is not merely not supernormal but is actually subnormal. Thus, the twenty embryos found in QT107 on the 16th day of pregnancy were all undergoing reabsorption, as were most of those found at a similar stage in QT109. QT143 and QT145–149 were laparotomized on the 11th day of pregnancy, and the numbers of implantations recorded in Table 6 are those observed at that stage. These five animals were allowed to go to term. QT143 and QT145 failed to produce litters, while QT146 produced five, QT148 three, and QT149 two living young. In view of the control results obtained by Parkes, Dodds & Noble [1938] in a similar kind of experiment, it is unlikely that the laparotomy had any influence in causing this holocaust of embryos, and it is most probably ascribable to abnormal conditions arising *in utero* from the implantation of supernormal numbers. In this connexion it may be recalled that Brambell [1942] has brought forward evidence showing that in wild rabbits the number of embryos which disappear entirely in the course of pregnancy is probably much greater than has hitherto been supposed.

The reasons why the uterus is unable to support the growth of supernormal numbers of embryos is not clear, but in view of the stage of pregnancy at which regression seems to have taken place in the experiments reported above, it is unlikely to be primarily of mechanical or metabolic origin. Further experimentation is clearly needed to determine whether abnormally large supplies of the hormones associated with pregnancy are required to support a superpregnancy, and, if so, whether the abnormally large number of corpora lutea present failed to produce abnormally large amounts of progesterone.

Induction of ovulation and pregnancy in immature rabbits

Three questions additional to those discussed above arise in connexion with any attempt to induce pregnancy in immature rabbits: (*a*) At what stage do the small follicles of the ovary become capable of responding to gonadotrophic stimulation by normal development? (*b*) If mating occurs when the follicles have been stimulated, will the pituitary body of the immature animal respond in the normal way by liberating ovulation-producing substance? (*c*) Will the pituitary body of the immature animal be capable of supporting the development of a functional corpus luteum after artificially or naturally induced ovulation, or will the continued administration of luteinizing hormone be necessary?

For the experiments described below Lop rabbits were selected, since their long period of immaturity provided more scope than would have been the case with Dutch or Himalayan. They were used at a body weight of 1·1–2·2 kg., the usual weight of rabbits of this breed at the first appearance of oestrus being about 3 kg. All the

Table 7. *Induction of ovulation in immature Lop rabbits primed with horse pituitary extract and then injected intravenously*

Number	Body weight g.	Time killed after i.v. injection days	Extract given intravenously		Condition of ovaries	Notes
			No.	Dose		
QT70	1300	1	AP25B	2 mg.	7 ovulation points	
QT68	1900	1	AP61B	2 mg.	6 ovulation points	
QT69	2100	1	AP61B	2 mg.	22 ovulation points	
QT67	Before puberty	3	AP61B	2 mg.	11 young corpora lutea in left ovary	Right ovary removed before i.v. injection
QT75	1500	5	UP10	10 i.u.	Some solid corpora lutea (?) from normal ovulations, also blood follicles and cystic follicles	Would not mate
QT133	1500	10	UP10	10 i.u.	Many corpora lutea, but seem small and pale for the stage	Uterus large
QT134	1500	10	UP10	10 i.u.	Ovaries large. Many normal corpora lutea	Uterus large
QT135	1600	10	UP10	10 i.u.	Ovaries small. No. of small white bodies, possibly atrophic corpora lutea	Uterus small
QT136	1600	10	UP10	10 i.u.	Ovaries small, but fairly normal corpora lutea	
QT132	1700	10	UP10	10 i.u.	Large number of normal corpora lutea. Ovaries large	Uterus large

animals were primed by five daily injections of 1 mg. of AP61B. In the first series of experiments intravenous injection was used to induce ovulation, and the rabbits were killed up to 10 days afterwards so that the development of the corpus luteum could be studied. The results show that even down to 1·3 kg. body weight follicular maturation and ovulation resulted from the treatment. Of the five rabbits left for 10 days after ovulation, however, only three had normal corpora lutea. In the remaining two, small pale bodies were found which were very similar to those described by Rowlands [1937] in rabbits injected after ovulation with anti-gonadotrophic serum, and which were almost certainly the remains of corpora lutea failing to develop owing to lack of endogenous luteinizing hormone.

In the next series of experiments, the priming process was followed by attempted mating without an ovulation-producing injection. Results are given in Table 8. It will be seen that all the rabbits down to 1·6 kg. body weight accepted the buck after priming. The two (QT72 and 78) which failed to mate were only 1·1 and 1·0 kg., and examination of the ovaries showed that there had been no morphological response to the priming process. The ovaries of QT78 weighed only 21 mg., but the uterus was much larger than usual in the immature Lop, very red, and obviously stimulated, so that there must have been some stimulation of the endocrinological function of the ovaries. Of those that mated, nine were examined after 5 days or less; all except one of those over 1·9 kg. ovulated, while two of 1·6 and 1·8 kg. failed to do so. Six were examined 9 or 10 days after mating; two showed no trace of having ovulated,

Table 8. *Induction of ovulation and pregnancy by mating of immature rabbits primed with horse pituitary extract*

Number	Body weight g.	Time killed after mating days	Result	Notes
QT78	1000	—	No obvious signs of stimulation in the ovaries	Killed day after last injection
QT72	1100	—	Ovaries very small. No growing follicles	Would not mate. Killed day after last injection
QT73	1600	1	No ovulation. Some large follicles and blood follicles	
QT71	2200	1	3 ovulation points. Several cystic follicles	
QT77	2100	2	No ovulation. Large numbers of nearly ripe follicles	i.v. injection of AP61B on the day after mating also failed to cause ovulation
QT80	1800	4	No ovulation	Ovary examined on day of mating. Large number of large follicles. Ovary much regressed by time of killing
QT137	1900	5	27 normal young corpora lutea	
QT138	2000	5	3 normal young corpora lutea	
QT140	2000	5	Large number of well-developed young corpora lutea, about 40 in all	
QT142	2000	5	Large number of well-developed young corpora lutea	
QT141	2100	5	18 young corpora lutea. Several large cystic follicles	
QT139	2200	5	10 normal young corpora lutea	
QT81	1900	9	Good corpora lutea in ovaries 3 days after mating. 15 atrophic corpora lutea in killing	
QT130	1500	10	Large ovaries with good corpora lutea	Pregnant. 20 embryos
QT127	1600	10	No good corpora lutea, but white patches which may be atrophic corpora lutea	
QT129	1600	10	Ovaries small. No corpora lutea. Doubtful if ovulated	
QT131	1600	10	Large ovaries with good corpora lutea	Pregnant. 21 embryos
QT128	1800	10	Ovaries small. No corpora lutea. Doubtful if ovulated	

and two had prematurely atrophic corpora lutea. Of these two, one was known from laparotomy to have had normal corpora lutea 3 days after mating. The two remaining rabbits had a large number of good corpora lutea, and had a supernormal number of embryos implanted. The correlation of these results with body weight was not good, since one of the rabbits that became pregnant was the smallest of the six, while one of those that failed even to ovulate was the second largest.

The only generalization that can be made from these experiments is that with the strain of Lop rabbits used premature ovulation and pregnancy can readily be induced by the time a body weight of 2·0 kg. is reached. At the other extreme, the follicular system is insensitive to gonadotrophic stimulation at a body weight of 1·0–1·1 kg. In between these body weights two different conditions are found; the follicles can

respond to priming but ovulation does not follow mating, or ovulation can take place but the corpora lutea do not develop. In other words, the chain of stimulation necessary to produce fertility in the immature rabbit can and does break at any of its links, but there is only a general correlation between the body weight of the animal and the stage at which breakdown occurs.

SUMMARY

1. Horse pituitary extract was used to increase the number of mature follicles in the ovaries of adult Albino, Lop, Dutch, Himalayan and Cross-bred rabbits.

2. The optimum duration of the priming process was found to be 5 days. Further injections evoked insensitivity to the extract which could not be overcome by a drastic increase of dosage.

3. The optimum daily dose of extract for priming was less in the small Dutch and Himalayan rabbits than in the other breeds.

4. Mating of the primed female caused ovulation of a number of follicles much in excess of the normal.

5. Maximal ovulation from the primed ovary could be obtained in any of the breeds without mating by the intravenous injection of 20 i.u. of chorionic gonadotrophin.

6. Superfecundation, as shown by the presence of large numbers of embryos at the end of the first third of pregnancy, was produced in many of the primed and mated rabbits, most of which also received an intravenous chorionic gonadotrophin injection to assist ovulation.

7. Superfecundated rabbits failed to produce an excessive number of young at term.

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CYCLICAL CHANGES IN THE SKIN OF THE MOUSE DURING THE OESTROUS CYCLE

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Studies by the author [Gibbs, 1938, 1941] have shown a correlation between the growth phases of the developing skin and hair follicles and those of the developing mouse ovary. The decrease in thickness of the epidermis, which takes place from the fourth to the thirteenth day, is followed by a phase of increase in thickness. This coincides with the first appearance of the follicular fluid in the ovary at the twelfth or thirteenth day which is followed by a period of rapid ovarian growth [Engle, 1931]. This fact suggested the possibility of a persistence of the relationship between variations in the thickness of the skin and the normal ovarian cycle. Such changes in the thickness are probably due to a variation in the mitotic activity of the epidermis, and it was considered possible that they might also be correlated with the cyclic mitotic activity of the ovary of the mouse which has recently been studied [Bullough & Gibbs, 1941; Bullough, 1942 *a*, *b*].

MATERIAL AND METHODS

The forty female mice used were identical with those used by Bullough [1942 *a*, *b*] in his study of the mitotic activity of the ovary. Several strains of mice were used, some of which had been previously mated. From birth they were kept at a constant temperature, exposed to 12 hr. of light daily, and fed on a standard diet. Vaginal smears were taken from each animal twice daily, and only those animals which had regular oestrous cycles were selected. The smears were stained in Ehrlich's haematoxylin and Pasini's stain which enabled the following stages of the oestrous cycle to be distinguished.

(1) Pro-oestrus: only blue-staining nucleated epithelial cells present.

(2) Oestrus:
- (*a*) pre-ovulation, cells cornified with cytoplasm stained in varying degrees of red;
- (*b*) early post-ovulation, cornified cells in clumps and stained red;
- (*c*) late post-ovulation, cornified cells largely degenerated and stained pale pink.

(3) Metoestrus: dark blue stained leucocytes scattered among pale pink cornified cells.

(4) Dioestrus:
- (*a*) first day, blue stained nucleated epithelial cells appear, leucocytes still most numerous, and traces of pale pink cornified cells persisting;
- (*b*) second and third days, only blue staining nucleated epithelial cells and leucocytes remaining.

The colchicine technique, as described by Allen [1937], was used to estimate the mitotic activity of the epidermis. Each animal was given a subcutaneous injection of 0·1 mg. of colchicine in 0·25 ml. of water 9½ hr. before death, so that all dividing cells were arrested in the metaphase during this final period. The mice were killed with chloroform, and the body cavity, thorax, and throat were opened. Each animal was dipped into 70 % alcohol as a wetting agent before being fixed for 2 days in Bouin's fluid. Small pieces of skin were then removed from the anterior and posterior dorsal regions, respectively midway between the bases of the fore and hind limbs. Each piece was trimmed into a rectangle along the line of the hair follicles, and after dehydration was immersed for 8 hr. in 58° C. m.p. wax, before being embedded in 52° C. m.p. wax. Sections were cut to a thickness of 7 μ. They were stained in Ehrlich's haematoxylin, and counter-stained in eosin and saffron.

Counts were made of the mitoses in the epidermis, lengths of 3 mm. being examined in twenty sections of skin from both the anterior and posterior dorsal regions of each animal. The arithmetic mean was calculated for each stage of the oestrous cycle in the two areas, and the standard deviation from the mean was found using the formula for small samples given by Simpson & Roe [1939].

The thickness of the epidermis and dermis was measured for both regions, and the average and standard deviation for each stage of the cycle was estimated.

In addition, sections of the ovary and uterus of identical animals, as used by Bullough [1942 *a*, *b*], were compared with sections of the skin, and their comparative mitotic activities assessed.

OBSERVATIONS

The structure of the epidermis and dermis of the mature female mouse is similar to that of young mice as already described [Gibbs, 1941].

The mitotic activity of the epidermis

The average number of mitoses per unit length in the anterior and posterior dorsal regions of the epidermis varied considerably throughout the oestrous cycle (see Table 1 and Fig. 1), and at every stage the anterior dorsal region showed the greater activity. In both regions the peak of activity occurred during pro-oestrus after which there was a slight decrease during pre-ovulation oestrus, followed by a sudden drop after ovulation. A slight rise in metoestrus preceded a period of very low activity

Table 1. *Average numbers of mitoses in the epidermis throughout the oestrous cycle*

Stage of oestrous cycle	Anterior dorsal			Posterior dorsal		
	a*	±	σ	a	±	σ
Pro-oestrus	548	±	169	240	±	174
Oestrus:						
Pre-ovulation	503	±	91	210	±	76
Post-ovulation, early	293	±	140	173	±	105
Post-ovulation, late	129	±	73	35	±	8
Metoestrus	152	±	49	109	±	26
Dioestrus:						
First day	89	±	111	50	±	53
Second day	223	±	150	98	±	82
Third day	238	±	50	84	±	57

* a = arithmetic mean. σ = standard deviation = $\sqrt{[fd^2/(n-1)]}$.

during the first day of dioestrus after which mitosis gradually increased throughout the second and third days of dioestrus to be followed once more by the sudden rise in pro-oestrus.

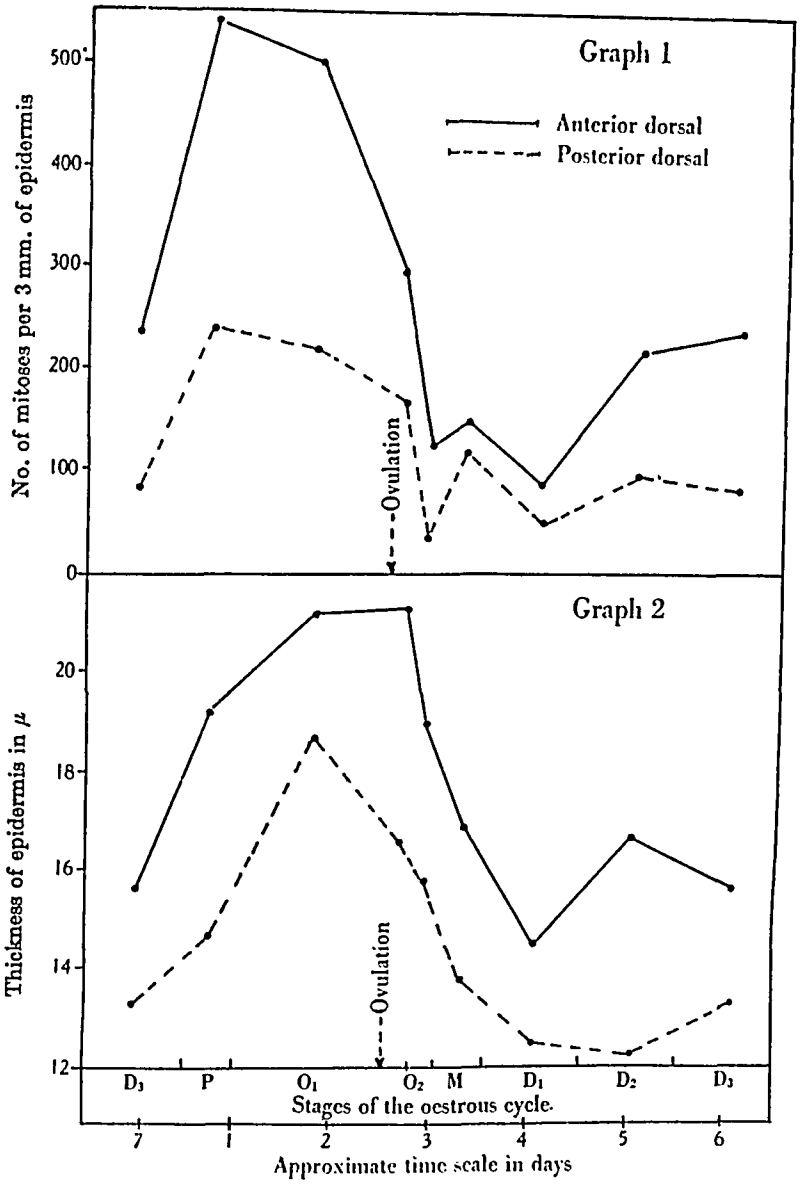


FIG. 1. Mitotic activity (above) and thickness (below) of the epidermis in relation to the oestrous cycle in the mouse.

The thickness of the skin

The epidermis. The average thickness of the epidermis in the anterior and posterior dorsal regions plotted against the stage of the oestrous cycle (Table 2 and Fig. 1) showed a distinct variation throughout the cycle. During pro-oestrus the epidermis gradually increased from one or two to four cell layers, and in late pro-oestrus indications of keratinization of the stratum corneum appeared. The highest point of

Table 2. *Average thickness of the skin (μ) throughout the oestrous cycle*

Stage of oestrous cycle		Thickness of epidermis			Thickness of dermis		
	n*	a*	±	σ*	a	±	σ
(*a*) *Anterior dorsal skin*							
Pro-oestrus	150	19·7	±	3·3	459·3	±	93·5
Oestrus:							
Pre-ovulation	200	21·2	±	4·6	341·3	±	89·6
Post-ovulation, early	200	21·3	±	4·8	456·7	±	139·5
Post-ovulation, late	200	18·9	±	4·1	529·7	±	119·5
Metoestrus	200	16·8	±	4·5	438·4	±	106·6
Dioestrus:							
First day	200	14·4	±	4·2	349·5	±	91·4
Second day	150	16·7	±	4·2	389·7	±	58·3
Third day	200	15·6	±	3·1	269·3	±	85·3
(*b*) *Posterior dorsal skin*							
Pro-oestrus	150	14·7	±	2·7	347·0	±	65·5
Oestrus:							
Pre-ovulation	200	18·7	±	3·9	391·3	±	204·0
Post-ovulation, early	200	16·6	±	4·3	357·4	±	194·4
Post-ovulation, late	200	15·5	±	3·4	336·1	±	172·8
Metoestrus	200	13·8	±	3·7	335·2	±	99·3
Dioestrus:							
First day	200	12·5	±	3·3	299·7	±	71·4
Second day	150	12·2	±	3·0	295·2	±	53·0
Third day	200	13·3	±	2·7	292·7	±	67·7

* n=number of measurements, a=arithmetic mean, σ=standard deviation=$\sqrt{[fd^2/(n-1)]}$.

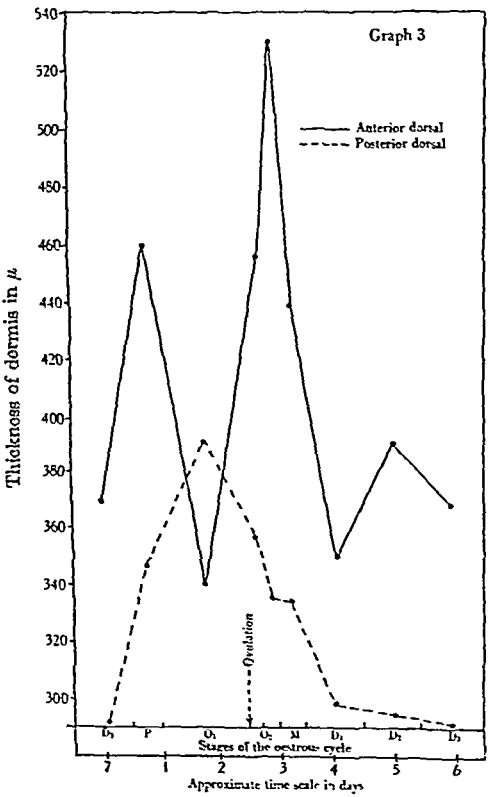


FIG. 2. Thickness of dermis in relation to oestrous cycle in the mouse

the curve in both regions occurred during pre-ovulation oestrus after the pro-oestrous period of intense mitotic activity. At this time the epidermis was four to six cell layers thick, and keratinization reached its climax with the production of a thick darkly staining stratum corneum. During post-ovulation oestrus the stratum corneum became separated from the remaining epidermal layers, and the thickness was reduced to about three cell layers. In both regions there was a marked decrease of thickness and lack of keratinization during the first day of dioestrus, which coincided closely with the low mitotic activity at that period. In fact, during dioestrus as a whole the epidermis remained comparatively thin and showed little increase until late pro-oestrus.

The dermis. For the purpose of these measurements (Table 2 and Fig. 2) the term dermis includes all tissues between the inner surface of the stratum cylindricum and the outer surface of the first layer of striated muscle. The measurement of the dermis presented difficulties in that its thickness varied considerably over a very short distance in any one animal. This great individual variation, as indicated by the standard deviation, seemed to suggest that any detailed study of the thickness during the cycle would be of little value. However, from the results obtained, there appeared to be a marked fluctuation in the thickness of the dermis from its highest point during oestrus to its lowest during dioestrus. This variation occurred almost entirely in the fatty layer, which apparently reached its greatest thickness during the later part of the oestrous period, and fell quite suddenly to a minimum during the first day of dioestrus.

A comparison with the ovary and uterus

In each animal sections of skin and uterus were compared at the various stages of the oestrous cycle, and it was found that the mitotic activity in the epidermis was invariably in direct relation to the mitotic activity of the uterus. For instance, the animal which gave the lowest mitosis count in the epidermis also showed the lowest activity in the uterus for that group. Similarly an animal of high epidermal activity would show great activity in the uterus.

Table 3. *Numbers of mitoses in the epidermis and ovarian germinal epithelium correlated with the numbers of Graafian follicles in mice killed in the pre-ovulation oestrous period*

No. of mouse	No. of Graafian follicles*	No. of mitoses in germinal epithelium*	No. of mitoses in epidermis (anterior dorsal)
13	2	32	84
251	3	62	223
14	4	101	475
131	5	107	430
12	6	93	472
83	7	149	673
346	11	201	180

* The figures for Graafian follicles and mitoses of the germinal epithelium were taken from Bullough 1942*a*].

Further, during the pre-ovulation oestrous period a close relationship was found to exist between the number of large Graafian follicles in the ovary and the mitotic

activity of the skin and germinal epithelium (Table 3). It will be noted that mouse no. 346 is an exception to this general rule, and this particular case will be discussed later.

DISCUSSION

Comparison of the graphs in Fig. 1 suggests a definite relationship between the rate of mitosis and the thickness of the epidermis. An examination of these graphs shows that the period of greatest mitotic activity during pro-oestrus is followed by a period of maximum thickness of epidermis during late pro-oestrus and pre-ovulation oestrus. The high rate of keratinization during late oestrus together with the gradual decrease in mitotic activity results in a decrease in thickness of the epidermis, the minimum activity and epidermal thickness occurring during the first day of dioestrus. With the renewed increase in mitotic activity in late dioestrus, a gradual increase in the thickness of the epidermis sets in.

In the case of the dermis, a detailed comparison of the results is more difficult. Even with the colchicine technique very few mitoses were observed. It seems possible to conclude, however, that a great increase in the fatty layer does occur during the oestrous period, while the minimum again occurs during dioestrus. By what means this fluctuation takes place it has not been possible to determine.

A comparison of the skin of the adult mouse with that of the young mouse and the Australian opossum

In the early post-natal development of the skin and hair of the Australian opossum, *Trichosurus vulpecula* [Gibbs, 1938], and of the mouse [Gibbs, 1941], phases of increase and decrease in thickness of the epidermis were noted which were then impossible to explain. It seems apparent now, however, that the phenomenon was due to a variation in the rate of mitotic activity in the epidermis. Further, in the work on the mouse some relationship seemed to be apparent between the developing ovary, as indicated by Butcher [1928] and Engle [1931], and the phases of development in the skin. In fact, Engle states that follicular fluid first appears in the ovarian follicles of the mouse at the twelfth or thirteenth day which coincides exactly with the time at which the period of decrease in the thickness of the epidermis ceases and an increase in thickness commences. Hargitt [1930] shows that irregular and incomplete cycles do occur in the ovary of the immature rat. It seems possible therefore that, as in the adult female, this variation in thickness of the epidermis in the young mouse is due to a change in the mitotic activity, which itself is in some way connected with an incomplete and immature ovarian cycle.

A correlation between the cyclic changes in the reproductive organs and in the skin

In the studies of oogenesis and its relation to the oestrous cycle in the adult mouse [Bullough & Gibbs, 1941; Bullough, 1942*a*] and the method of follicle development in the ovary of the mouse [Bullough, 1942*b*] animals identical with those of the present work were used, and interesting comparisons resulted. For instance, the lowest point of mitotic activity in the germinal epithelium of the ovary was found to occur during the first day of dioestrus, which coincides with the time of lowest mitotic activity in the mouse epidermis. On the other hand, the peak of activity of the germinal epithelium occurs later than it does in the epidermis. Further,

during the pre-ovulation period of oestrus there appears to be some connexion between the large maturing follicles then present in the ovary, the number of mitoses in the germinal epithelium, and in the epidermis (Table 3). In fact, it seems apparent that the number of mitoses in the germinal epithelium and in the skin varies directly with the number of large follicles in the ovary. The exception would appear to be animal no. 346, which, although it had the largest number of Graafian follicles, gave a low count for mitoses in the epidermis. This may be explained by the fact that the animal appeared to be actually on the point of ovulation, for Bullough [1942*b*] has shown that at this time the mitotic activity of the ovarian follicle and stroma cells is considerably reduced, and it may be that the same condition exists in the epidermis. Bullough also found a slight increase in mitosis in those cells of the new corpus luteum which lie adjacent to the new but short-lived reservoir of follicular fluid appearing during late oestrus or early metoestrus. This activity coincides with a slight rise in mitotic activity in the epidermis (Fig. 1), and might well be due to a similar cause.

Further evidence of the close connexion between the mitotic activity of the skin and that of the reproductive organs was indicated by a comparison of sections of epidermis and uterus of identical animals. The mitotic activity of the epidermis was found to be invariably strictly proportional to that of the uterus.

Epidermal activity during the oestrous cycle in other animals

Apart from the many specialized studies on the sexual skin of monkeys, the only previous work done on changes in the normal skin during the oestrous cycle appears to be that of Loeb & Haven [1929] on the guinea-pig. Apparently they did not use either the vaginal-smear technique or the colchicine method. Consequently, the stages of the oestrous cycle were not well defined, and the numbers of cells in mitosis were found to be very small. However, they state that mitotic activity is much reduced during the oestrous period, that there is a slight rise from the first to the fifth day after heat, a temporary rise from the seventh to the tenth day, and that the greatest activity occurred on the fifteenth and sixteenth days of the cycle just before a new opening of the vagina was observed. This would probably correspond to pro-oestrus, the period of greatest activity in the mouse skin. It is difficult, however, with the incomplete data given, to make any closer correlation between the two animals. The statement of Loeb & Haven that the greatest mitotic activity occurs in the guinea-pig when neither the corpus luteum nor the follicular hormone is functioning seems to be denied by the present work and by that of Bullough, for it is now shown that the greatest activity in the epidermis, uterus, and ovarian follicle and stroma cells occurs when there is a large and rapidly growing accumulation of follicular fluid in the ovary.

GENERAL CONCLUSION

The conclusion is reached that a definite connexion exists between the cyclic growth of the reproductive organs and that of the skin, and the suggestion is made that the causative factor in both cases may be the same.

SUMMARY

1. The mitotic activity of the epidermis in the mouse (estimated by means of the colchicine technique) varies throughout the oestrous cycle, the maximum activity occurring during pro-oestrus and the minimum during the first day of dioestrus. During pro-oestrus and early oestrus the mitotic activity of the epidermis varies directly with the number of maturing follicles in the ovary, and it also shows a direct relationship with the mitotic activity of the uterus.

2. The thickness of the epidermis fluctuates with the rate of mitotic activity, being greatest in early oestrus (just after the pro-oestrous period of maximum mitotic activity) and least on the first day of dioestrus.

3. Great individual variation occurs in the thickness of the dermis, but there appears to be an increase in the thickness of the fatty layer during oestrus.

My thanks are due to Professor E. A. Spaul for his advice and criticism of the manuscript, and to my husband, Dr W. S. Bullough, for his constant help and co-operation. I also wish to thank the donors of the Ackroyd Memorial Fellowship for the opportunity of carrying out this investigation.

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RATE OF ABSORPTION OF ESTERS OF OESTRONE AND OESTRADIOL AS DETERMINED BY FEATHER TESTS

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The well-known response of the breast feathers of the Brown Leghorn capon to oestrogens can readily be used to determine the rate of absorption of different esters [Parkes, 1937]. In the male the breast feathers are black; injection of oestrogen while feathers are growing causes deposition of the fawn pigment characteristic of the female. Only that part of the feather is changed which is growing when oestrogen is present; existing plumage is not affected. Since the rate of feather growth is known, the width of the fawn bar in the black feather indicates the time during which effective amounts of oestrogen were circulating. Thus, if a single injection is given, the duration of effect of a compound can easily be ascertained.

In the work previously recorded the duration of action of the following substances was examined: oestrone, oestrone acetate, oestrone benzoate, oestrone methyl ether, oestradiol, oestradiol diacetate, oestradiol monobenzoate, oestradiol dibenzoate, oestradiol 3-benzoate 17-acetate, 17-methyl oestradiol, oestriol, oestriol triacetate. The results were clear cut, and showed, in concordance with mammalian experiments, that certain of the esters, notably oestradiol benzoate acetate, have a very prolonged action. It was later demonstrated [Deanesly & Parkes, 1937] that the prolonged action is due to delayed absorption from the site of injection.

Following the earlier work, experiments were carried out with a large series of aliphatic esters of oestrone and oestradiol, and, after unavoidable delay, the results are now recorded.

TECHNIQUE

The technique was as previously described. Capons were used in groups, usually of five. One side of the ventral surface of each bird was stripped of feathers, and about 10 days later when the new feathers appeared through the skin, the substance to be tested was injected intramuscularly, dissolved in 1 ml. of arachis oil. All doses were calculated in terms of free hormone. About 6 weeks later when feather growth was complete, representative feathers were plucked from each bird, mounted, and the width of the bar measured to the nearest millimetre. Where the bar was highly irregular, or where, as with medium doses of some of the higher esters, the response took the form of a blotch or a thick rachis stripe, measurement was not attempted. Under the conditions of maintenance of the birds, the breast feathers grew at a rate of about 2 mm./day [Parkes, 1937] so that the 'duration of effect of the hormone in days' was obtained by dividing by two the 'width of the bar in mm.'

RESULTS

Oestrone

The results for the oestrone esters are shown in Table 1. The following comments may be made.

(1) In general, the results entirely confirm those obtained on rats by Miescher, Scholz & Tschopp [1938*a*]. These authors showed that with the aliphatic esters of oestrone, the duration of the response of the ovariectomized rat was directly proportional to the number of carbon atoms in the acid chain, and that with the higher esters the minimum effective dose was much increased.

Table 1. *Oestrone esters*

Ester	Dose mg.	No. of capons	Average width of bar mm.	Average duration of response days
Propionate	0·25	5	2·4	1
	1·0	4	3·7	2
	4·0	5	5·8	3
n-Butyrate	0·25	5	2·0	1
	1·0	5	4·2	2
	4·0	5	5·6	3
iso-Butyrate	0·25	4	3·5	2
	1·0	4	8·0	4
	4·0	5	9·0	4
Valerate	0·1	4	3·7	2
	0·25	5	6·2	3
	1·0	5	14·6	7
	4·0	5	17·2	9
Caproate	0·1	5	2·8	1
	0·25	5	7·4	4
	1·0	5	20·2	10
Caprylate	0·1	5	5·0	2
	0·25	5	7·4	4
	1·0	—	22·4	11
Laurate	0·1	4	Blotchy or nothing	—
	0·25	5	Blotchy or nothing	—
	0·5	4	20·2	10
	1·0	3	32·6	16
Palmitate	0·1	4	Blotchy or nothing	—
	0·25	5	Irregular rachis marks	—
	0·5	4	Considerable but very irregular response	—
	1·0	4		—

(2) In the feather test, a single injection of 1 mg. of free oestrone causes a response for only about 2 days. Increase of dosage does not prolong the response. The acetate is but little more effective [Parkes, 1937]. It will be seen from Table 1 that the propionate and the *n*-butyrate are similar to each other in duration of effect and both are not obviously better than the acetate or the free substance. The *iso*-butyrate, however, is much better and the improvement continues up to the caprylate. In this series the intensity of the response, as judged by the minimum effective dose, is not decreased, but actually increases. The laurate and the palmitate, however, although giving a very prolonged action with the higher doses, give a highly irregular response with the

lower doses, the fawn area being in the nature of a blotch on the rachis rather than of a transverse bar. This type of response, similar to that previously described for sub-effective doses of oestradiol dibenzoate, is caused by prolonged minimal dosage.

Table 2. *Oestradiol esters*

Ester	Dose mg.	No. of capons	Average width of bar mm.	Average duration of response days
Di-propionate	0·05	5	3·8	2
	0·1	5	8·0	4
	0·25	5	11·4	6
	1·0	5	24·6	12
3-Benzoate-17-butyrate	0·05	5	0	0
	0·1	4	0	0
	0·25	5	Blotches or nothing	—
	1·0	3	40·3	20
17-Propionate	0·1	4	1 only with bar	0
	0·25	2	3·5	2
	1·0	5	4·6	2
17-Caprylate	0·05	5	Rachis stripes or nothing	—
	0·1	5	Rachis stripes or blotchy	—
	0·25	4	Blotchy	—
	1·0	4	40+	20+

Oestradiol

The original paper [Parkes, 1937] recorded results in feather experiments with three esters of oestradiol, the diacetate, 3-benzoate, and the 3-benzoate-17-acetate. Data for the dibenzoate were added by Deanesly & Parkes [1937]. Four further esters have now been investigated in the feather test, the di-propionate, the 3-benzoate-17-butyrate, the 17-propionate and the 17-caprylate (Table 2). The results are substantially in agreement with those obtained by Miescher *et al.* [1938*b*] in experiments on ovariectomized rats. The dipropionate proved to be a highly effective ester, having an action both more intense and more prolonged than the 3-benzoate. The 3-benzoate-17-butyrate had a low intensity, being inactive in the lower doses, but a very prolonged effect in the higher doses, and was generally similar in action to oestrone laurate and palmitate. The 17-propionate was similar to oestrone propionate in being but little better than the free hormone. The 17-caprylate gave a very irregular response in the lower doses and a very prolonged one in the higher doses, being similar in this respect to oestradiol dibenzoate and to the higher esters of oestrone.

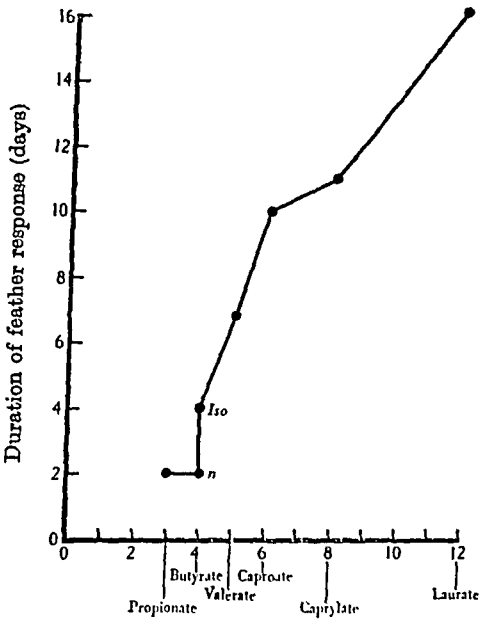


FIG. 1. Duration of feather response to 1 mg. of oestrone in esterified form in relation to number of carbon atoms in the acid chain.

On the basis of these experiments, it seems that esterification in position 17 has more effect in delaying absorption than has the same modification in position 3.

SUMMARY

1. The duration of action of further esters of oestrone and oestradiol has been tested by ascertaining the time for which, following a single injection, they will feminize the ventral feathers of Brown Leghorn capons.

2. The results are in full agreement with those previously obtained in the feather test by Parkes [1937] and with those obtained on rats by Miescher *et al.* [1938].

3. Deanesly & Parkes [1937] found that the prolongation of action caused by esterification was due in all cases examined to delay of absorption from the site of injection, and it is likely that a similar explanation applies to the esters discussed in the present paper.

I am much indebted to Messrs Ciba, and the British Drug Houses respectively, for the esters of oestradiol and oestrone.

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THE ROLE OF THE ADRENAL CORTEX AND ANTERIOR PITUITARY GLAND IN INDUCED SECONDARY SHOCK SYMPTOMS

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(*Received 10 August 1942*)

In recent years there has been considerable discussion about the influence of adrenal cortical extracts on secondary shock. While some observers hold that treatment with such extracts has a distinct influence on the severity of shock produced by various means, others have expressed the opinion that adrenal cortical extracts have little or no value in either the prevention or treatment of shock.

The present paper is not only concerned with the effect of adrenal extracts on animals suffering from a shock-like syndrome, but also with the effect of anterior pituitary hormones on such animals, as well as with the effect of these two principles on adrenalectomized and hypophysectomized animals in a shock-like state.

Our method of producing a shock-like state (intraperitoneal injections of hypertonic glucose solution) does not claim to cause traumatic shock but does reproduce a number of symptoms which also occur in the clinical syndrome of traumatic shock. This method would appear to have the advantage of a reasonably quantitative gradation of the stimulus and is, thus, amenable to more rigid experimental control.

EXPERIMENTAL

The method chosen was designed to allow on the one hand the production of alterations of body temperature, oxygen consumption and haemo-concentration simulating as closely as possible those observed in secondary shock, and on the other hand to be capable of quantitative variation. Thus it would be possible by suitable adjustment of the conditions to decide whether the resistance of an animal had been decreased or increased by any particular treatment.

It was found that if a hypertonic (40 %) solution of glucose were injected into the peritoneal cavity of our Wistar rats, the following changes would develop after half an hour.

(1) A sharp reduction in the oxygen consumption.

(2) A marked decline in body temperature.

(3) Concentration of the blood, as shown by an increase in the percentage of red cells determined by haematocrit.

(4) A fall in blood sodium content.

(5) A fall in total plasma protein.

These alterations were susceptible to gradual variation, the temperature falling further and the concentration of the blood increasing as greater quantities of glucose were injected. When the amount of glucose solution employed reached the value of 4 ml./100 g. body weight, the rats, after 1 hr., lay in the cage in a drowsy and finally

prostrated condition, convulsions frequently occurred and they died 2½–3 hr. after the injection. It was noticed that the breathing first became very slow and finally stopped about a full minute before the heart ceased to beat. Another point of interest is that immediately after death the capillaries were seen to be markedly dilated throughout the peritoneal cavity. Although the effect on plasma proteins has not yet been fully investigated, preliminary experiments have shown a diminution of plasma protein of about 30% compared with that of normal rats of the same weight. With smaller doses normal animals generally survived after a marked fall in temperature, haemo-concentration and other symptoms of secondary shock. In many experiments it was possible to demonstrate a direct relation between the intensity of the measurable effects and the dose of glucose solution injected.

Dehydration shock has previously been studied by Davis [1940 *a*, *b*, 1941], though his experiments were chiefly directed to the investigation of histological changes. He noted, however, that dehydration leads also to deficient oxygenation of the tissues and increased permeability and dilatation of the capillaries, resulting from the local effect of poorly oxygenated blood.

Body temperature

The use of mercury thermometers for temperature measurement was soon abandoned, a thermo-electric method being used for the later series of experiments. The rectal temperature was measured by means of a thermo-element protected by a thin

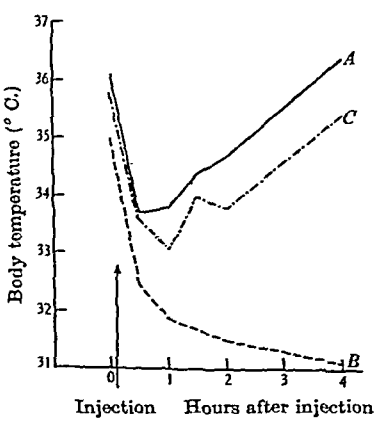


FIG. 1.

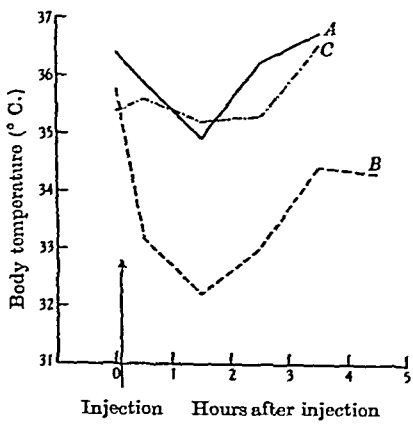


FIG. 2.

FIG. 1. Fall in temperature in infantile male rats (36–40 g.) injected intraperitoneally with 1 ml. of 40% glucose solution. *A*, normal controls (6 animals). *B*, adrenalectomized 3 days previously (6 animals). *C*, adrenalectomized and pretreated with desoxycorticosterone acetate 3 days previously (6 animals).

FIG. 2. Effect on body temperature in male rats (83–135 g.). *A*, normal controls (7 animals). *B*, adrenalectomized 2 days previously (7 animals). *C*, adrenalectomized and pretreated with corticotrophic hormone 2 days previously (7 animals).

steel tube of 2 mm. diameter and 2 cm. length, the animals being kept throughout the experiment in a room maintained at 25° C. The results of these experiments are shown in Figs. 1–4; Fig. 1 showing average temperature curves from the following

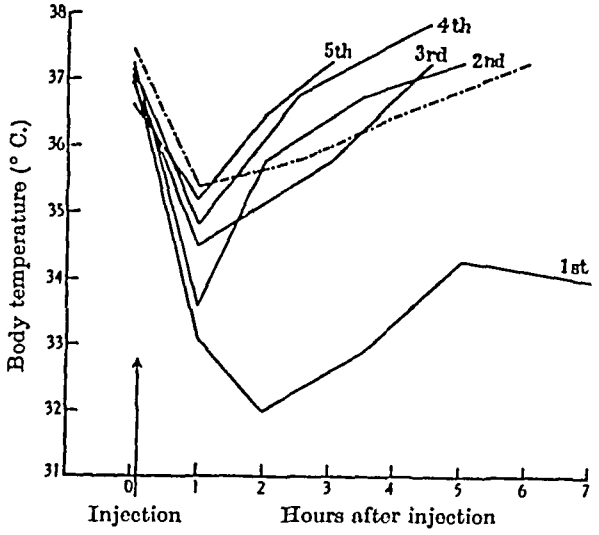


FIG. 3. Body temperature in adult male rats (250–300 g.) following successive intraperitoneal injections of hypertonic glucose solution. —— 32 animals, serial intraperitoneal injections of 5 ml. of 40 % glucose solution. Numbers on curves show order of injections on alternate days. —·— 12 animals pretreated with corticotrophic hormone injected intraperitoneally with 5 ml. of 40 % glucose solution.

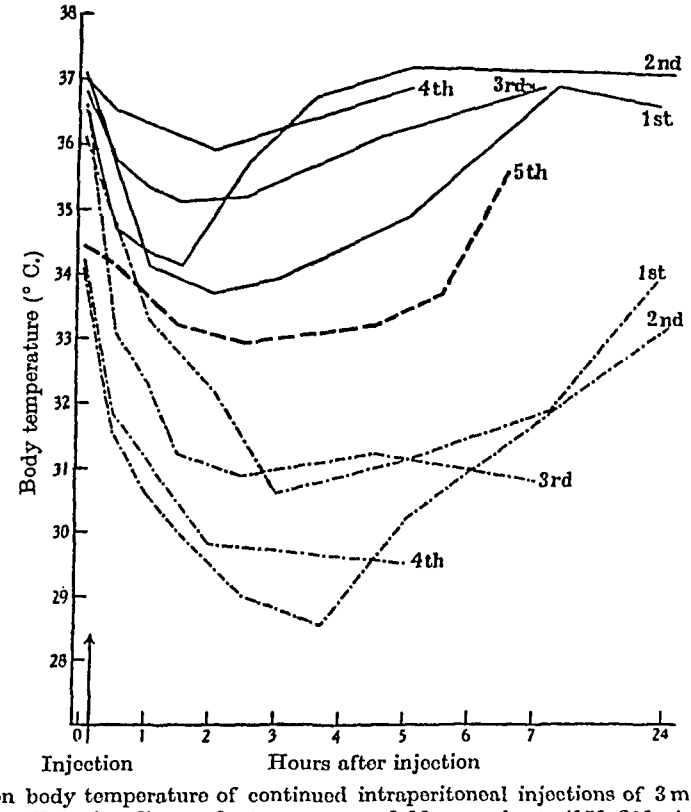


FIG. 4. Effects on body temperature of continued intraperitoneal injections of 3 ml. of 40 % glucose solution. —— Unbroken lines refer to a group of 12 normal rats (150–200 g.) given 4 injections at 2-day intervals. —·— Broken lines refer to a group of 12 hypophysectomized animals which received serial injections as in the normals. Between 2nd and 3rd injections, 4 animals died, 2 more between 3rd and 4th injections, leaving 6 survivors. —— Heavy broken lines show results following glucose injection after treating the 6 surviving hypophysectomized animals with corticotrophic hormone for 2 days. All curves represent the average temperatures of the animals used in each case.

three groups of animals: (*a*) normal untreated controls of 36–40 g. weight; (*b*) animals of the same age and weight adrenalectomized 3 days previously; (*c*) animals as in (*b*) but treated beforehand with 1 mg. of desoxycorticosterone acetate. All animals in each of the three groups received the same amount of 40 % glucose. The normal controls showed the characteristic fall of temperature but recovered after 4 hr. The animals of group (*b*), which were adrenalectomized but otherwise untreated, showed a greater fall of temperature, failed to recover and died. The group (*c*) animals treated with desoxycorticosterone acetate did not die and behaved like the normal controls in group (*a*).

Fig. 2 shows the results of an experiment similar in nature to that of Fig. 1 except that heavier rats were used and the amount of glucose was therefore smaller in proportion. It is clear that the adrenalectomized animals showed a much greater fall in temperature and a slower recovery. The animals of group (*c*) were treated in advance with corticotrophic hormone of the anterior pituitary gland and, although adrenalectomized, showed on the average a smaller temperature fall and an even more rapid recovery than the untreated controls of group (*a*).

Fig. 3 shows the average temperature curves obtained from a group of thirty-two normal rats of 250–300 g. body weight. These rats were treated on alternate days with the relatively larger amount of 5 ml. of 40 % glucose injected intraperitoneally. After the first injection they showed an average fall of temperature to 32° C., and even after 7 hr. had not completely recovered. After the second injection the fall of temperature was less, to 36° C., recovery being complete after 5 hr. From the diagram it can be seen how the resistance of the rats increased with successive injections. When after six injections these animals were killed in order to carry out chemical determinations on the blood (see Table 2), it was found that the average weight of the adrenals had increased by 55 %, and that of the pituitaries by 20 %, as compared with the corresponding organs in the untreated control group kept under similar conditions.

In the same figure are shown the results obtained on twelve animals of similar weights kept under the same conditions, but treated with corticotrophic hormone 3 days prior to the glucose injection. Such animals displayed a decidedly lower fall in temperature than normal rats, following the first injection, and behaved like rats the resistance of which had been increased by previous shocks.

Fig. 4 illustrates the differences in behaviour as regards temperature disturbance, between groups of normal and hypophysectomized rats when repeated injections of 3 ml. of 40 % glucose were administered intraperitoneally at 2-day intervals. Whereas the twelve intact animals used showed the development of resistance to the temperature-lowering effect of the injections, the hypophysectomized rats tended to become, if anything, more sensitive, and some, in fact, died following the later glucose injections. After four injections had been given on alternate days, the six rats surviving from the original group of twelve were given corticotrophic hormone for 2 days and were again injected with glucose. The result, as shown by the average temperature course following the injection, was a definite improvement, though not a return to the behaviour of the normal controls. The results, based on average figures, seem to show with fair clearness that hypophysectomy not only makes the animals more sensitive, but also reduces the power to develop resistance. The effect of corticotrophin would seem to suggest that the adrenal cortex plays an important part in these phenomena.

Oxygen consumption

The oxygen consumption of the rats used was measured in a closed system as in earlier experiments [Reiss, 1941], employing spirometers. A few characteristic results are given in Fig. 5. It can be seen that the oxygen consumption of untreated rats is diminished 1 hr. after injection of the hypertonic glucose solution. The fall in oxygen

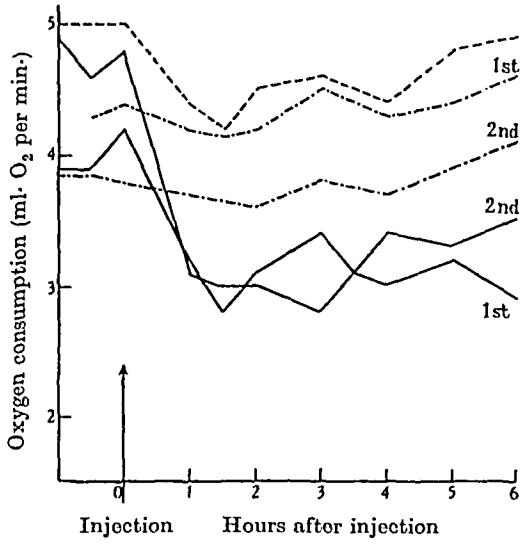


FIG. 5. Oxygen consumption of rats injected intraperitoneally with 2·5 ml. of 40 % glucose solution. —— *Normal controls (2 animals).* – – – – *Animal injected subcutaneously with 20 units of corticotrophic hormone ½ hour before glucose injection.* —·— Animals subcutaneously injected with 50 units of corticotrophic hormone for 2 days preceding the experiment (2 animals).

uptake is distinctly lower in the case of an animal which had received corticotrophic hormone half an hour before the glucose injection. Results were not always uniform, due presumably to variations in the rate of absorption of the hormone and the susceptibility of the adrenal cortex. If the animals were treated with corticotrophic hormone 2 days previously, the depression of the oxygen consumption following glucose injection was negligible.

Chemical changes in the blood

Blood was obtained during decapitation, heparin being used to prevent coagulation. Sodium was determined according to Weinbach's method [Weinbach, 1935]. For haematocrit determination average values were taken from ten glass capillaries each time sealed at one end after admitting blood. Results are shown in Tables 1–3 and display changes occurring in the same sense as in the experiments discussed in the two sections above. The blood of adrenalectomized animals following glucose injection shows a higher red cell percentage and a lower sodium value than controls. Treatment with corticotrophic hormone tends to inhibit both of these reactions, a similar inhibitory effect being obtained with desoxycorticosterone.

Treatment with corticotrophic hormone tends to prevent to a considerable extent the occurrence of such changes even in normal animals. The sodium falls less and the cell volume rises less. Table 2 shows also the blood chemical changes in the animals

Table 1

Body weight of animals g.	Treatment before experiment	Treatment during experiment	No. of animals used	Haematocrit arithmetic mean ± σ %	Blood sodium mg. % arithmetic mean ± σ	Changes: Haematocrit %	Changes: Blood sodium %
80–100	Adrenalectomized 3 days previously	Untreated	3	45·8 ± 0·984	248 ± 14·8		
		3 hr. before bleeding 2·5 ml. 40 % glucose (intraperit.)	4	54·1 ± 0·835	149 ± 12·3	+20·2	−40
	Adrenalectomized 3 days previously and treated with 15 u. corticotrophic hormone 2 × daily	Untreated	3	48·1 ± 0·936	236 ± 13·2		
		3 hr. before bleeding 2·5 ml. 40 % glucose (intraperit.)	4	51·3 ± 0·748	184 ± 15·1	+ 6·6	−22
	Controls	Untreated	3	45·4 ± 1·13	284 ± 10·1		
		3 hr. before bleeding 2·5 ml. 40 % glucose (intraperit.)	4	52·3 ± 0·684	232 ± 9·2	+15·2	−18
60–80	Adrenalectomized 2 days previously	Untreated	4	42·8 ± 0·982	302 ± 18·1		
		1½ hr. before bleeding 1·5 ml. 40 % glucose (intraperit.)	8	55·9 ± 0·935	173 ± 11·0	+30·6	−43
	Adrenalectomized 2 days previously and treated with 15 u. corticotrophic extract 2 × daily	Untreated	4	40·9 ± 1·22	274 ± 8·3		
		1½ hr. before bleeding 1·5 ml. 40 % glucose (intraperit.)	7	50·1 ± 0·551	255 ± 12·1	+24·9	− 7
	Controls	Untreated	4	40·5 ± 0·928	296 ± 13·4		
		1½ hr. before bleeding 1·5 ml. 40 % glucose (intraperit.)	4	43·9 ± 0·385	277 ± 7·3	+ 8·4	− 6
70–100	Adrenalectomized 2 days previously	Untreated	2	45·8 ± 0·750	286 ± 9·1		
		2 hr. before bleeding 1·5 ml. 40 % glucose (intraperit.)	3	55·7 ± 0·352	176 ± 5·2	+21·6	−39
	Adrenalectomized 2 days previously and treated with 1 mg. desoxycorticosterone acetate daily	Untreated	2	30·6 ± 0·55	261 ± 10·2		
		2 hr. before bleeding 1·5 ml. 40 % glucose (intraperit.)	5	49·4 ± 0·485	266 ± 9·1	+24·6	+ 2
	Adrenalectomized 2 days previously and treated with 15 u. corticotrophic extract	Untreated	3	46·4 ± 0·634	262 ± 11·8		
		2 hr. before bleeding 1·5 ml. 40 % glucose (intraperit.)	4	49·7 ± 0·792	253 ± 4·8	+ 7·3	− 3
	Controls	Untreated	3	44·9 ± 0·854	281 ± 16·2		
		2 hr. before bleeding 1·5 ml. 40 % glucose (intraperit.)	5	51·6 ± 0·996	267 ± 10·2	+14·8	− 5

Table 2. *Experiments on animals of* 250–300 *g. kept under identical conditions of diet and room temperature before and during the experiment*

Treatment before experiment Average weights of adrenals and pituitaries after killing	Controls untreated throughout experiment: No. of animals used	Haematocrit arithmetic mean ± σ %	Blood Na mg. % arithmetic mean ± σ	Animals injected with 5 ml. 40 % glucose intraperitoneally: No. of animals used	Hr. before bleeding	Haematocrit arithmetic mean ± σ %	Blood Na mg. % arithmetic mean ± σ	Changes: Haematocrit %	Changes: Blood sodium %
Untreated Adrenals: 35 mg. Pituitaries: 10·1 mg.	12	42·8 ± 1·24	242 ± 12·1	5	½	59·3 ± 0·812	164 ± 14·2	+38·6	−32
				12	1½	63·2 ± 0·728	143 ± 10·1	+47·6	−41
				6	3	60·0 ± 0·566	157 ± 12·4	+40·0	−36
				5	4½	59·6 ± 0·488	152 ± 9·2	+39·1	−37
				6	6	56·2 ± 0·512	170 ± 10·1	+33·6	−29
Animals injected 5 times (intraperitoneally) on alternate days with 5 ml. 40 % glucose before experiment Adrenals: 54 mg. Pituitaries: 12·2 mg.	6	41·9 ± 0·638	247 ± 14·2	5	½	50·2 ± 0·935	198 ± 10·9	+19·8	−20
				5	1½	52·3 ± 0·855	194 ± 13·9	+24·4	−21
				6	3	49·8 ± 0·735	209 ± 10·8	+18·8	−16
				6	6	44·3 ± 0·636	266 ± 6·4	+5·7	+ 8
Animals injected (subcutaneously) 2 × daily with 15 u. corticotrophic hormone for 3 days	6	46·6 ± 0·616	250 ± 10·1	6	1	52·0 ± 0·588	185 ± 6·5	+11·5	−26
				12	6	50·2 ± 0·936	204 ± 10·2	+ 7·7	−18

Table 3. *Experiments on animals of* 150–200 *g. kept under identical conditions of diet and room temperature before and during the experiment*

No. of animals used	Treatment for 2 days before experiment	Treatment during experiment	Haematocrit arithmetic mean ± σ %	Blood Na mg. % arithmetic mean ± σ	Changes: Haematocrit %	Changes: Blood sodium %
(*a*) *Normal rats*						
4	Controls	Untreated	41·1 ± 0·994	284·6 ± 15·4	—	—
4	Controls	5 hr. before bleeding 3 ml. 40 % glucose intraperitoneally	48·7 ± 0·863	260·4 ± 12·1	+18·6	− 8·5
6	1 mg. desoxycorticosterone acetate daily	,, ,,	47·1 ± 1·12	263·5 ± 14·8	+14·6	− 7·4
6	5 u. corticotrophic hormone 2 × daily	,, ,,	44·8 ± 0·634	254·4 ± 10·2	+ 9·0	−10·2
6	Pituitary total extract containing 10 u. gonadotrophic, 5 u. thyrotrophic, 3 u. corticotrophic hormone 2 × daily	,, ,,	43·0 ± 0·786	243·5 ± 12·8	+ 4·6	−14·4
(*b*) *Completely hypophysectomized rats*						
4	Controls	Untreated	43·4 ± 0·662	283·9 ± 18·5	—	—
4	Controls	5 hr. before bleeding 3 ml. 40 % glucose intraperitoneally	50·1 ± 0·793	186·2 ± 9·6	+15·6	−34·4
4	1 mg. desoxycorticosterone acetate daily	,, ,,	51·9 ± 0·968	229·9 ± 11·8	+19·6	−19·0
6	5 u. corticotrophic hormone 2 × daily	,, ,,	42·7 ± 0·989	244·8 ± 14·5	− 1·6	−13·7
5	Pituitary total extract (as above) 2 × daily	,, ,,	46·3 ± 0·836	250·3 ± 16·7	+ 6·7	−11·8

previously referred to in Fig. 2. These, after the sixth glucose shock, showed a smaller diminution of sodium and a smaller rise in cell volume than the control group, shocked for the first time.

The blood changes studied appear to follow a somewhat similar course when hypophysectomized instead of intact animals are used for the experiments. Table 3 shows that the fall in blood sodium is more pronounced in hypophysectomized than in normal rats, and that after treatment with corticotrophic hormone or total anterior lobe extracts this effect disappears. Desoxycorticosterone acetate was less effective than the two former preparations in diminishing the rise in relative blood-cell volume.

DISCUSSION

The introduction of a solution so strongly hypertonic as that employed in our experiments undoubtedly provokes a far-reaching upset of equilibrium between the capillaries and the neighbouring interstitial tissues, and this in turn may involve damage to the endothelial function, which may be either transitory or permanent, depending on the particular experimental conditions. Several authors have demonstrated that a characteristic feature of secondary shock is a disturbance of capillary permeability, leading to loss of a large part of the circulating plasma proteins. The kind of damage to the endothelial function which is envisaged might be likened to that described by Zweifach [1941] who examined the effects of trauma produced in parts remote from those investigated (tongue and mesentery of the frog). He was able to show that first of all the contractile response to nerve stimulation vanishes, with the result that the capillaries remain partly dilated, and as a consequence the venous circulation is slowed and the permeability of the vessels increased.

Following adrenalectomy the permeability of membranes is known to be altered. Thus, the rate of absorption of sodium, potassium and chloride from loops of the ileum is diminished in adrenalectomized dogs [Denis & Wood, 1940]. Remington [1940] found large differences in absorption rates between normal and adrenalectomized animals following intraperitoneal injection of isotonic glucose and sucrose solutions in large amounts (10% of the body weight). The increase in permeability produced by inflammatory exudates can be inhibited wholly or in part by adrenal cortical hormone [Menkin, 1940]. The relationship between capillary function and adrenal cortical hormone is made abundantly clear in a recently published paper [Rhoads, Wolff & Lee, 1941] dealing with burn shock, where it is shown that adrenal cortical hormone enables the vessels to retain a greater proportion of transfused plasma protein.

Our own experiments have demonstrated the close relationship existing between the adrenal cortex and the anterior pituitary, on the one hand, and the intensity of the shock-like syndromes produced by intraperitoneal injection of hypertonic glucose solution, on the other. The increase of resistance produced in adrenalectomized, but especially in normal, animals by desoxycorticosterone and still more by corticotrophic hormone is a powerful argument in support of this claim. The fact that the resistance of adrenalectomized rats could also be increased by corticotrophic hormone can only be explained on the assumption that the corticotrophic hormone in this case stimulates accessory cortical tissue, which, in its turn, produces sufficient cortical hormone to increase the resistance. We may further assume that the increased

resistance occurring in normal animals following repeated glucose injections depends upon increased endogenous production of corticotrophic hormone and, consequently, of adrenal cortical hormone. This explanation is supported by the increase in weight of the adrenals and pituitaries actually observed as well as by the failure to develop resistance in hypophysectomized animals. This would be in line with the observation of Weil & Browne [1939] that there is an increased secretion of adrenal cortical hormone in patients exposed to surgical shock.

Further experiments are in progress to examine the effect of total anterior pituitary gland extract and desoxycorticosterone upon the behaviour of hypophysectomized rats after intraperitoneal glucose injection. As far as can be judged at present, desoxycorticosterone acetate seems capable of protecting those animals to a certain extent, while a total extract of the anterior lobe is probably slightly more effective than a partially purified preparation with only corticotrophic action.

The following points might be stressed as resulting from the present investigation. First, by the use of corticotrophic hormone the full activity of the suprarenal cortex can be mobilized very rapidly; Fig. 5 illustrates that an effect can actually be demonstrated within half an hour of injection. This may be compared with the rapid action of gonadotrophic hormone on the ovaries [Reiss, Druckrey & Fischl, 1932] and of thyrotrophic hormone on the thyroid gland [Reiss, Hochwald & Druckrey, 1933]. Secondly, the body's own adrenal cortex is stimulated by corticotrophin as contrasted with a substitution therapy when cortin or desoxycorticosterone acetate is given. The latter, in any case, is in all probability not the only secretory substance of the adrenal cortex; in fact it is even doubtful if desoxycorticosterone is secreted by the adrenal cortex at all. Selye & Dosne [1940] claim that desoxycorticosterone acetate is ineffective against traumatic shock, corticosterone only being of value. The latter substance, however, has not yet been prepared synthetically and is not available in any quantity. Apart from this, there are theoretical objections to the use of desoxycorticosterone or cortin in that they depress the functions of the body's own adrenal cortex. Finally, our results suggest that it may be worth while to investigate the effect of corticotrophic preparations (which are fairly easily accessible) in early stages of secondary shock. An investigation of the prophylactic use of these substances in cases where secondary shock is to be anticipated may also be of interest.

SUMMARY

Shock-like symptoms (decrease in oxygen consumption, decrease in body temperature, rise of blood haematocrit values and blood sodium content) were produced in rats by intraperitoneal injections of a hypertonic glucose solution. This method may permit an approximately quantitative gradation of the intensity of the shock-like condition.

The sensitivity of adrenalectomized and hypophysectomized animals to such shock was found to be remarkably increased.

Corticotrophic hormone, and to a lesser extent desoxycorticosterone, were able to increase the resistance to the effects of intraperitoneal glucose injections not only of adrenalectomized and hypophysectomized animals but also of normal animals.

The resistance against the development of the shock-like symptoms produced by our method in normal animals was found to be increased after repeated injections of

hypertonic glucose solutions. Hypophysectomized animals, on the other hand, failed to acquire such resistance.

The possible clinical significance and application of these results are discussed.

Our thanks are due to Messrs Organon Ltd., London, for the gift of generous supplies of desoxycorticosterone acetate and corticotrophic hormone. Acknowledgement is also due to W. Gross, H. Peglar and E. Graetzer for much valuable technical assistance.

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THE EFFECT OF VARIOUS HORMONES ON THE CHEMICAL AND PHYSICAL PROPERTIES OF BONE

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Clinicians have long been familiar with diseases of the endocrine system in which alterations of bone shape occur, and chemical investigations have now shed some light on these conditions. Of all hormones the growth-promoting factor of the anterior pituitary gland is undoubtedly the most potent in inducing skeletal change. The pituitary may exert a direct control over bone growth and the effects of other hormones—e.g. those of the thyroid and parathyroid glands and the gonads—may have to be explained on the basis of their effects on the production of this growth-promoting factor. On the other hand, the converse may hold, viz. the action of the pituitary may be indirect.

While there is considerable quantitative information about the gross metabolic changes in bone disease, there is little or no information about the nature of the physicochemical alterations, if any, produced [Logan, 1940]. It is well known that there is an increase in the percentage of calcium in the bones with increasing age, but apart from this the analyses of bones in health and disease are very much alike. X-ray spectrography [Reynolds, Hayden & Corrigan, 1938] has shown that the apatite structure is maintained in health and disease and that hyperparathyroidism is the only condition in which an alteration has been detected.

We have shown already [Bell, Cuthbertson & Orr, 1941] that rats on a very low calcium intake produced very thin-walled bones, which were relatively weak, but in spite of this the bones were still of normal shape. Generalized calcium deficiency, therefore, does not appear to be the essential cause of deformity. Since this work showed that we now possess a satisfactory method of investigating bone strength it seemed to us of interest to apply it to the bones of animals which had been treated with various hormones and to inquire if this new technique would throw any light on the nature of the bony changes in diseases of the endocrine system.

METHODS

The animals used were white male rats. They were all fed on an adequate diet [Thomson, 1936], and the hormones were administered either with the food in the case of the thyroid or injected subcutaneously in the case of the other hormones. The controls of the same age received no treatment except in the case of the control animals of the pituitary experiments which received thymus extract instead of pituitary extract. The methods of feeding and the methods of chemical analysis were exactly as we have described in our previous paper.

Measurement of bone strength

The methods employed to measure the strength of the bones have already been described in considerable detail [Bell *et al.* 1941], and only a brief summary will be given here. Consideration of the tests of strength of materials available to engineers showed that only two, viz. bending and twisting tests, were likely to represent the straining actions taking place in the living animal. For the *bending* test each right femur had its ends cast into semicylindrical pieces of hard resin. With the femur supported in a horizontal position weights were added slowly to a pan hanging from the centre of the shaft until the bone broke. If the distance between the points of support is l, and the breaking load is W, then the bending moment M at the mid-point of the bone where it breaks is $Wl/4$; this is an index of the strength of the bone. The quality of the bone material can be judged from the breaking stress s_1 derived from

$$M = s_1(abt), \tag{1}$$

or

$$M = s_1\left(\frac{\pi}{32}\frac{ab^3 - dc^3}{b}\right), \tag{3}$$

where a, b, c, d, t refer to the dimensions of the central section of the bone (Fig. 1). For the *twisting* test hollow cubes were cast on the ends of the left femora and one cube was held fixed. Increasing loads were applied in small steps to a pan hung on a

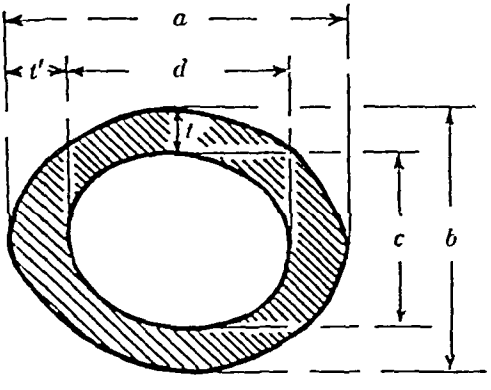


FIG. 1. Diagrammatic section through the mid-point of the shaft of the femur to indicate where the measurements were made. Reproduced by courtesy of *The Journal of Physiology*.

lever attached to the other end of the bone (at right angles to its long axis) until the breaking point was reached. The twisting moment $T = Wl$, where l is the length of the lever; this is a measure of the strength of the bone. The breaking stress on twisting, s_2, an index of the quality of the bone material, is calculated from

$$T = s_2(abt). \tag{2}$$

Formulae (1) and (2) do not give absolute values but the figures obtained are comparable among themselves. Formula (3) provides an absolute value for the breaking stress.

Anterior pituitary extracts

The extracts of the anterior lobe of the pituitary gland were supplied by Dr F. G. Young who prepared them by grinding the fresh frozen anterior lobes in cold saline at *p*H 8·5 [Young, 1938]. This extract has, in addition to its growth-promoting activity, thyrotropic, diabetogenic, glycotropic, gonadotropic and prolactin activities.

The animals of group 1 received 1 ml. of this extract equivalent to 0·125 g. of fresh ox anterior pituitary per day; the controls received a similar extract of calf thymus gland. In group 2 each ml. of extract was equivalent to 0·25 g. of fresh anterior pituitary; animals 1–5 received 0·2 ml./day of horse pituitary extract, 6–10 the same amount of sheep pituitary extract, 11–15 the same amount of ox pituitary extract, while 16–20 (control animals) received the same dosage of an extract of thymus. The animals of group 3 received 0·5 ml. of crude alkaline extract of ox anterior pituitary gland (1 ml. ≡ 0·25 g. of fresh tissue), the controls receiving the same quantity of an extract of calf thymus. This experiment lasted 82 days, in comparison with the 25- and 12-day periods of groups 1 and 2.

Oestradiol

The animals were given 0·1 mg. of oestradiol dipropionate (Ovocyclin P, Ciba) at the beginning of the experiment and again 3 weeks later. This can be considered a very large dose of oestrogenic material, since it has been shown by Miescher, Scholz & Tschopp [1938] that half this dose would produce an oestrus lasting 40 days in castrated female rats. Gardner & Pfeiffer [1938] found that endosteal bone nearly or completely replaced the marrow cavities of the femora of mice which had received 250 i.u., or more, of oestrogens weekly over long periods (about a year).

Parathyroid hormone

Ten units of parathyroid hormone (Parathormone, Lilly) were given for the first 3 days of the experiment, and 3 units daily for the next 38 days—total dose 144 units to each animal. This dosage is nearly the same as that given to rats by Reynolds, Corrigan, Hayden, Macy & Hunscher [1938] over a slightly shorter period (31 days).

Thyroid

In the first thyroid experiment 10 grains (0·67 g.) of thyroideum siccum B.P. were incorporated in each 100 g. of food; the average intake of thyroideum siccum was approximately 0·12 g./day.

A batch of thyroid-treated animals which had been used for another experiment not requiring controls was given us. For comparison we selected animals of approximately the same original weight from our stock. In this second experiment the amount of thyroid in the food had been so adjusted that the animals received 0·1 g. thyroideum siccum per day.

RESULTS AND DISCUSSION

The detailed results of the experiments are grouped together in Table 1; and the more important findings are presented graphically in Figs. 2 *a*, *b*.

Table 1

Treatment	No. of animals	Duration of exp. days	Initial body wt. g.	Final body wt. g.	Approx. food intake g.	Length of body cm.	Length of femur cm.	Wt. of femur g.	Ca content of femur g.	% Ca in air-dried femur
Group 1:										
Ox anterior pituitary	10	25	68	175	—	—	2·99	0·310	0·075	24·2
Calf thymus controls	9	25	68	144	—	—	2·80	0·266	0·066	25·0
Group 2:										
Horse anterior pituitary	5	12	176	210	—	—	3·15	0·440	0·125	28·4
Sheep anterior pituitary	5	12	182	216	—	—	3·27	0·424	0·130	30·6
Ox anterior pituitary	5	12	182	236	—	—	3·30	0·430	0·116	27·0
Calf thymus controls	5	12	164	176	—	—	3·14	0·377	0·118	31·4
Group 3:										
Ox anterior pituitary	10	82	61	286	—	—	3·73	0·604	0·181*	30·0*
Calf thymus controls	10	82	61	194	—	—	3·43	0·495	0·149*	30·0*
Oestradiol dipropionate	7	41	248	289	31	20·6	3·62	0·546	0·146	26·7
Parathyroid hormone	7	41	245	329	36	21·4	3·71	0·597	0·162	27·2
Controls	5	41	233	289	31	20·8	3·63	0·517	0·146	28·2
Group 1:										
Thyroid	4	29	256	222	18	19·1	3·54	0·616	0·165	25·2
Controls	4	29	259	292	22	20·3	3·56	0·613	0·177	28·9
Group 2:										
Thyroid	6	76	253	208	30	18·3	3·32	0·435	0·115	26·5
Controls	3†	—	—	260	—	20·0	3·54	0·535	0·147	27·5

Treatment	Total Ca in carcass less gut. g.	% Ca in carcass	*M* in. lb.	Breaking stress M/abt Formula (1)	Breaking stress $32/\pi$ etc. lb./in.² Formula (3)	*T* in. lb.	Breaking stress T/abt Formula (2)	Dimensions of femora 0·001 in. *a*	*b*	*t*
Group 1:										
Ox anterior pituitary	1·49	0·85	2·60	13·67	30,000	1·83	9·85	124·5	97·5	15·6
Calf thymus controls	1·28	0·89	2·79	15·62	35,550	1·58	7·84	119·1	93·7	15·9
Group 2:										
Horse anterior pituitary	2·09	1·00	3·63	14·47	34,500	2·01	8·12	123·5	100·5	20·2
Sheep anterior pituitary	2·19	1·07	4·29	15·60	35,800	2·55	9·50	127·3	103·7	20·7
Ox anterior pituitary	2·26	0·96	3·98	15·10	36,160	2·23	8·79	128·8	101·5	19·5
Calf thymus controls	2·05	1·16	3·49	16·20	36,300	2·15	10·00	124·3	96·9	18·1
Group 3:										
Ox anterior pituitary	3·16*	0·91*	6·56	15·69	33,530	4·08	10·70	151·6	123·7	21·4
Calf thymus controls	2·47*	0·97*	5·67	16·13	37,360	2·63	8·07	142·7	112·6	20·9
Oestradiol dipropionate	2·76	0·96	5·39	15·43	34,270	3·72	10·54	152·7	111·6	21·0
Parathyroid hormone	3·06	0·93	5·26	14·68	32,070	3·62	11·38	148·2	110·1	20·9
Controls	2·78	0·96	4·96	15·07	34,020	3·26	11·29	150·0	107·7	20·6
Group 1:										
Thyroid	2·57	1·16	4·38	14·45	32,530	2·86	10·23	134·9	111·0	20·2
Controls	2·76	0·94	4·94	15·39	31,300	3·05	9·87	140·3	112·7	20·6
Group 2:										
Thyroid	2·38	1·93	3·41	13·43	31,300	2·25	9·14	128·9	101·2	19·6
Controls	2·96	1·33	3·90	12·37	28,000	3·22	9·60	139·6	111·5	20·3

* Some of the data for the femora are missing. The calculations are based on the assumption that percentage of calcium in air-dried femur is 30.
† Stock animals of the same original weight.

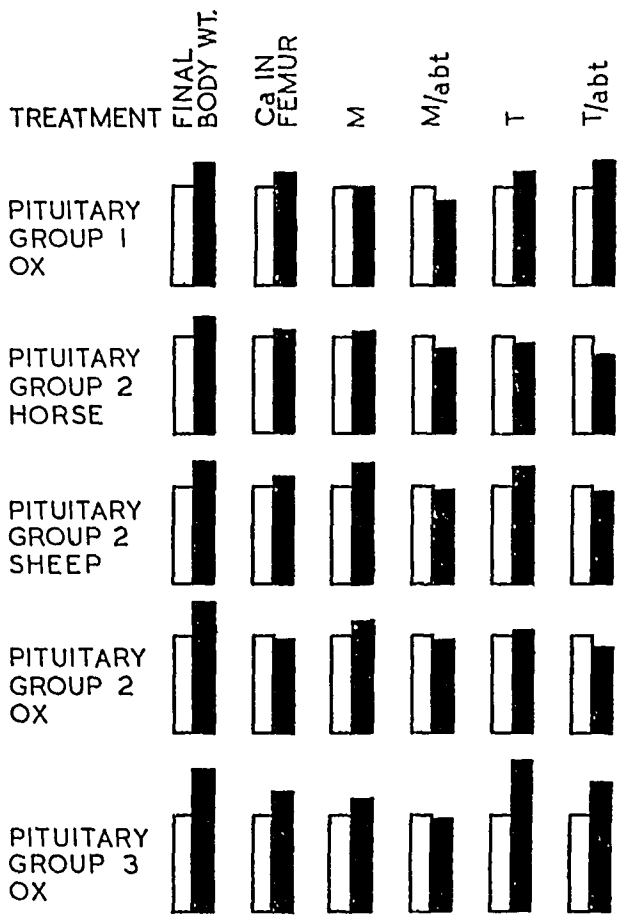
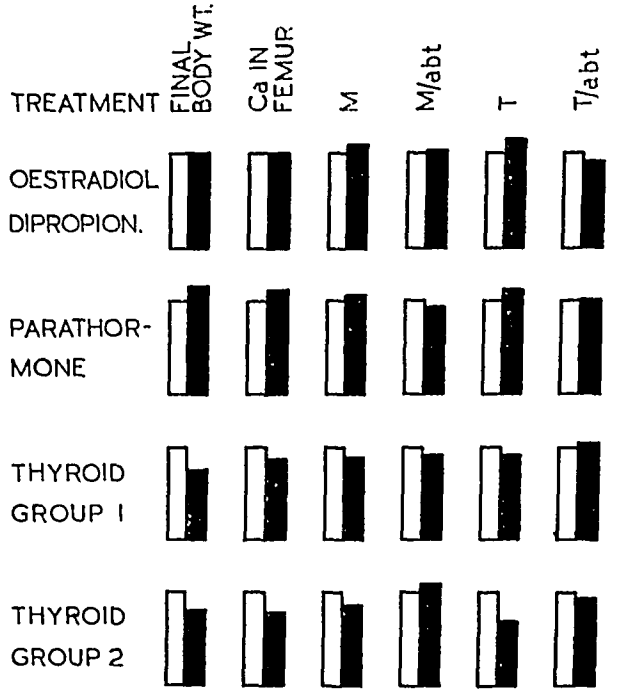


FIG. 2*a*. Some of the more important information relative to the influence of the anterior pituitary, extracts expressed diagrammatically. The findings in the control animals have been taken as 100 % (clear rectangles) and have been compared with the data for the treated animals (dark rectangles).



Pituitary experiments

It will be seen at once that the anterior pituitary extracts all accelerated growth very considerably, the ox pituitary extract being the most potent. The treated animals were all heavier, had heavier bones, and had more calcium in their bodies than the controls. The amount of calcium in the femora was either the same or very little increased so that the percentage of calcium in the bone was slightly smaller in the treated groups; the same difference is found in young rapidly growing bones when they are compared with older bones. The bones of the pituitary-treated groups were almost always stronger than the control bones—indeed, the greatest differences between treated and control groups in the entire series have been produced by treatment with anterior pituitary extracts. The increase in strength is, however, accounted for almost entirely by the increase in dimensions; the figures for breaking stress suggest that there may actually be a small diminution in the quality of bone material, but they do not consistently point in this direction. (The quality of the bone is indicated by the breaking stress which has been calculated from formulae (1), (2) and (3).)

Oestradiol experiments

It will be seen that we were unable to produce any considerable alteration in the size of the femora or their calcium content or in the total and percentage calcium in the carcass, nor were we able to reproduce the striking reduction of the marrow cavity described by Gardner & Pfeiffer [1938]. It may be either that rats are more resistant than mice to skeletal changes or that our experiments were not sufficiently prolonged.

The bones of the oestradiol-treated animals were slightly stronger than those of the controls (see *M* and *T*), but this is completely accounted for by their slightly larger dimensions so that the actual quality of the bony material indicated by formulae (1), (2) and (3) is the same.

Parathyroid experiments

Reynolds *et al.* [1938] found on X-ray analysis of the bone powder of their parathyroid-treated animals a pattern differing slightly from that given by the controls, but they did not offer any interpretation of the difference. It is rather surprising that there should be any changes in rat bones because these animals—judged by clinical and chemical tests—are almost immune to chronic high dosage of parathyroid hormone. Selye [1932] has drawn attention to the stimulating effect of this hormone on the formation of osteoblasts leading to bone apposition. Human beings are also fairly resistant to chronic high dosage, but in spite of this, changes have also been reported in the X-ray spectrogram in cases of hyperparathyroidism [Reynolds *et al.* 1938].

There was a marked increase in the body weight of the parathyroid-hormone-treated animals as compared with the controls; they were also slightly longer and had bigger and heavier femora indicating increased osteoblastic activity. The percentage of calcium in the femur was very little affected by parathyroid hormone treatment; this was also the case in the experiments of Reynolds *et al.* [1938]. The total calcium of the carcass was higher than that of the controls although the percentage of calcium was, if anything, less; thus while the hormone induced a greater deposition of bone there was a nearly equivalent addition of soft tissue.

The femora of the parathyroid group were slightly stronger than those of the controls but since the dimensions of the former were proportionately greater the quality of the bone material in both groups is the same.

Thyroid experiments

The first experiment was the more severe; the animals lost on the average 1·2 g./day as against 0·6 g. in the second experiment. In the first and shorter experiment the treated animals lost 13% of their body weight as against 18% in the second experiment which lasted more than two and a half times longer. The gain in weight of the control animals of the first experiment was 13%; the total body loss of the thyroid-treated animals was therefore of the order of 26%. There is thus no doubt that the dosage of thyroid was sufficient to produce a very profound disturbance of metabolism; indeed in such a chronic experiment the dosage could not safely be increased. In these circumstances if skeletal changes accompany the hyperthyroid state then they certainly should have appeared in our experiments.

The loss of skeletal calcium was not quite proportional to the general loss in body weight since the percentage of calcium in the thyroid carcasses was greater than in the controls. In the second more chronic experiment there was little difference in the percentage of calcium in the femur, but in the more severe first experiment there was a definite reduction. It is of interest that the skiagrams in group 1 showed no difference between treated and control bones although there was a 13% reduction in the calcium of the femora. This indicates that X-ray evidence is only of value after considerable resorption of bone has occurred.

The strength of the thyroid-treated bones was less in both experiments but in both cases the bones were reduced in size in all dimensions so that the quality of the bone material was the same in all animals.

CONCLUSIONS

In these experiments we have attempted to induce changes in the metabolism of bone by means of hormones. Certain quantitative changes in the strength of the bones have obviously resulted, but these can be practically completely accounted for by the alterations produced in the dimensions of the bones so that the actual quality, or breaking stress, is substantially the same as that of the controls.

This work offers no explanation of the change of shape seen in bone diseases produced by excessive action of certain hormones. Measurements of the deflexion of the centre of the femur during the bending tests were made on representative samples of all the groups (2 from the oestradiol group, 2 from the parathormone group, 2 from the thyroid group, 4 from the pituitary group and 10 from the corresponding controls). There was no difference between controls and treated bones; in all cases the graph of deflexion against load was a straight line up to the breaking point exactly as in the graph already published. It may be wiser to seek the causes of the deformities which are seen clinically in terms of the reaction of the bony tissue to muscular pull or body weight—an abnormal variant of the normal modelling process—rather than in terms of a qualitative alteration of the osseous tissue.

The results described do not permit of any useful speculation with regard to the riddle of direct or indirect action of the pituitary hormone on bone growth. Indeed,

the most striking point about the present experiments and those previously undertaken on calcium deficiency is the constancy of the physical and chemical characteristics of bone—a feature which is presumably of value from the point of view of the survival of the organism. It may be—although a wider field must first be covered—that we shall have to give up the idea that there are bones of varying quality, just as it has been found necessary to abandon the theory that in disease an endocrine organ may produce an abnormal secretion.

SUMMARY

The administration to rats of a crude alkaline extract of the anterior pituitary gland with growth-promoting properties produced bigger and proportionately stronger bones than those found in controls injected with thymus extract, but there was no alteration in the quality (breaking stress) of the bony material laid down.

Oestradiol dipropionate induced the formation of slightly heavier and stronger bones than normal, but again their strength in bending and twisting tests indicated no qualitative change.

The femora of parathyroid-treated animals were slightly longer and heavier than those of either oestradiol-treated or untreated control rats. The strength tests again did not show any difference in quality.

Thyroid gland fed to rats produced a more marked reduction in soft tissue than in mineral matter; but although this was considerable the quality of the bone did not appear to have deteriorated.

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PRODUCTION OF OVULATION IN HYPOPHYSECTOMIZED RATS

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In the intact rat the intrinsic action of gonadotrophic substances is often complicated by the action of the endogenous gonadotrophins of the test animal. Evidence of such complications is provided by histological differences between the condition of the ovaries of intact and hypophysectomized immature rats when subjected to stimulation by each of a variety of different gonadotrophic substances [Noble, Rowlands, Warwick & Williams, 1939], and by the ability of mare serum gonadotrophin to cause ovulation in intact rats but not in hypophysectomized rats [Rowlands & Williams, 1941]. The ineffectiveness of the serum gonadotrophin in causing ovulation in the absence of the pituitary gland of the test animal may be partly explained by its relative lack of luteinizing activity, but it is also certainly caused, in part, by the atrophic condition of the ovaries when the injection is made [Williams, 1943].

Leonard & Smith [1933] observed that in hypophysectomized rats an extract prepared from the urine of post-menopausal women caused only follicular stimulation; its action thus resembled that of mare serum gonadotrophin. Ovulation did not occur unless the rats were subsequently treated with chorionic gonadotrophin. This latter substance, by itself, does not cause ovulation in the hypophysectomized rat. Foster and his colleagues [Foster & Fevold, 1935; Foster, Foster & Hisaw, 1937] have shown that ovulation may also be produced in hypophysectomized rabbits if follicle-stimulating and luteinizing extracts are both given, but not if either of them is given alone.

Guided by these results we have produced ovulation and even superovulation in hypophysectomized rats by overcoming the ovarian atrophy with a single injection of mare serum gonadotrophin and subsequently injecting chorionic or other gonadotrophins. We have worked out in some detail the optimal conditions for producing ovulation by these means, so that the results so far obtained may act as a basis for similar investigations in other species.

MATERIAL AND METHODS

Hypophysectomy. Immature, or in some cases mature, female rats were hypophysectomized by the retro-pharyngeal approach under ether anaesthesia. Body-weight changes and inspection of the sella turcica at autopsy served as checks on the completeness of the operation.

Extracts. The following gonadotrophic preparations were used and were made up in aqueous solution before injection.

(*a*) Mare serum gonadotrophin: PMS 26—a sample of dried serum which was reconstituted by the addition of water before injection. The resulting solution contained 200 international units (i.u.) per ml.

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(*b*) Chorionic gonadotrophin: UP31—an extract of human pregnancy urine containing 200 international units (i.u.) per mg.

(*c*) Sheep pituitary gonadotrophin: AP117B.

(*d*) Pig pituitary gonadotrophin: AP74D.

(*e*) Ox pituitary gonadotrophin: $AP32D_1$.

The three pituitary extracts were prepared by fractionation of the acetone-desiccated pituitary tissue [Chance, Rowlands & Young, 1939] and the gonadotrophic effects of similar extracts in intact and hypophysectomized rats have been described [Noble *et al.* 1939].

Injections. Each preparation was administered as a single subcutaneous injection. The preliminary injection of mare serum gonadotrophin was made 7–9 days after hypophysectomy by which time the ovaries were atrophic. The other gonadotrophins were subsequently injected as described below.

Criteria of ovulation. In the early stages of this work the occurrence of ovulation was determined by examination of serial sections of the Fallopian tubes for the presence of ova. Later a quicker and more economical method was adopted [Rowlands, 1942]. The Fallopian tubes were unravelled in the fresh condition under a binocular dissecting microscope. When superovulation had occurred that segment of the tube containing the ova surrounded by cumulus cells was usually sufficiently swollen and translucent to be recognized by the naked eye. When the tube contained only a small number of ova no swelling was visible, but under the microscope the eggs were easily seen. This segment was then excised, its contents expressed and the ova counted.

RESULTS

Passage of ova through the Fallopian tubes

Five days after the injection of 40 i.u. of mare serum gonadotrophin the ovaries of the hypophysectomized immature rat weigh 20–25 mg. and contain numerous mature follicles, but no corpora lutea. The ovaries are, therefore, in an approximately similar condition to those of an intact rat at puberty. Groups of hypophysectomized immature rats were injected with 40 i.u. of serum gonadotrophin and again 5 days later with either 0·5 mg. or 1 mg. of the sheep pituitary extract, AP117B; such an extract is known to have predominantly luteinizing activity [Noble *et al.* 1939]. The rats were killed at intervals from 12 to 96 hr. after the injection of pituitary extract and the Fallopian tubes were examined. The results are given in Table 1.

Ovulation had obviously occurred between the 12th and 24th hr. after the injection of AP117B. The number of rats that had ovulated in the two groups killed at the 24th hr. was proportional to the dose of AP117B received, though the difference in dose had no appreciable effect on the number of ova discharged from the ovaries. With the lower dose there was, in the following days, a progressive fall in both the proportion of tubes containing ova and in the number of ova that each contained. The fact that this decline was not so rapid with the higher dose of AP117B suggests that its action was more prolonged and that further ovulation may have occurred after the 24th hr. Finally, it is clear that all ova had passed through the tubes, or had disintegrated, by the 96th hr. after the injection of the pituitary extract. The

time taken by the ova to pass through the Fallopian tubes of these rats is, therefore, similar to that of the eggs of intact rats discharged into the tube at a normal ovulation [Huber, 1915].

Table 1. *Passage of ova through Fallopian tubes of immature hypophysectomized rats injected with mare serum gonadotrophin and sheep pituitary gonadotrophin* (AP117B)

Time of killing after injection of AP117B hr.	Amount of AP117B injected mg.	No. of rats	No. of rats with ova in tubes %	Average no. of ova per rat
12	0·5	—	—	—
	1·0	6	0	0
24	0·5	5	40	11
	1·0	7	86	10
48	0·5	8	25	5
	1·0	6	83	12
72	0·5	6	17	1
	1·0	5	40	4
96	0·5	9	0	0
	1·0	8	0	0

Gonadotrophic extracts producing ovulation

Other gonadotrophins were then tested for their ability to produce ovulation in immature hypophysectomized rats which had already received 40 i.u. of serum gonadotrophin. The experimental procedure was exactly similar to that adopted above with the sheep pituitary gonadotrophin. All the animals were killed 24 hr. after the second injection. The results given in Table 2 show that, under these conditions, ovulation can be produced with chorionic gonadotrophin, pig pituitary gonadotrophin and even by a second injection of serum gonadotrophin itself. We failed to produce ovulation with ox pituitary extract in the dosage used.

Table 2. *Ovulation in hypophysectomized rats injected with various gonadotrophins following an injection of mare serum gonadotrophin*

Gonadotrophin	Dose	No. of rats	Ovulation %	Average no. of ova per rat
Chorionic, UP31	10 i.u.	6	0	0
	50 i.u.	7	100	29
Mare serum, PMS26	8 i.u.	7	0	0
	40 i.u.	6	17	15
	200 i.u.	7	86	16
Ox pituitary, AP32D$_1$	5·0 mg.	4	0	0
Pig pituitary, AP74D	2·5 mg.	6	83	14
	12·5 mg.	6	66	5
Sheep pituitary, AP117B	0·5 mg.	5	40	11
	1·0 mg.	7	86	10

The number of ova produced was generally greater than the number which would be expected in the Fallopian tubes after ovulation in intact untreated rats. This was especially the case with chorionic gonadotrophin which was the only exclusively luteinizing extract used. The average number of ova produced was 29, a number which corresponds very closely to that given by Rowlands [1943] for ova released into the tubes of intact immature rats after receiving similar treatment. The maximum

number produced in any one rat was 49. In view of this high incidence of superovulation, chorionic gonadotrophin was used in all the following experiments.

Optimal interval between injections

The optimal interval between the injections of serum gonadotrophin and chorionic gonadotrophin was determined in hypophysectomized rats weighing 100–120 g. The rats in this experiment were injected, as before, with 40 i.u. of serum gonadotrophin 7 days after hypophysectomy and were killed 18 hr. after the injection of chorionic gonadotrophin. The optimum interval was found to be 4 days, which is, presumably, the time required for the serum gonadotrophin to bring the ovarian follicles from a condition of atrophy to one of maturity. If the injection of chorionic gonadotrophin is postponed until the 5th day a smaller number of rats ovulate; it is probable that by this time atresia of the ripened follicles has already set in. The difference between the two results, given in Tables 2 and 3, produced when 50 i.u. of chorionic gonadotrophin are given 5 days after the serum gonadotrophin is probably due to the difference in size of the rats used. The same dose of gonadotrophin will obviously produce a higher concentration in the blood of the smaller rats.

Table 3. *The effect on ovulation of the interval between injections of serum gonadotrophin and chorionic gonadotrophin* (UP 31)

Interval between injections days	Dose of UP 31 i.u.	No. of rats	Ovulation %
3	50	10	40
	100	11	45
4	50	10	80
	100	12	92
5	50	19	42
	100	10	50

Time of ovulation

A further experiment was performed to determine more accurately the time of ovulation after the injection of chorionic gonadotrophin. In this instance 50 i.u. of chorionic gonadotrophin (UP 31) were injected 4 days after a preliminary injection of 40 i.u. of serum gonadotrophin. Immature hypophysectomized rats were used and the serum gonadotrophin was injected 7–9 days after the operation. The animals were killed 12–17 hr. after the injection of UP 31. The results, given in Table 4, show that

Table 4. *Time of ovulation in hypophysectomized rats injected with mare serum gonadotrophin and chorionic gonadotrophin* (UP 31)

Time killed after UP 31 injection hr.	No. of rats	Rats with ova in tubes %	Time killed after UP 31 injection hr.	No. of rats	Rats with ova in tubes %
12	6	0	14½	9	89
13	8	0	15–16	17	88
13½	11	36	16–17	18	94
14	13	54			

ova are first found in the Fallopian tubes in the 13th and 14th hr. after the injection of the luteinizing substance. It is obvious that ovulation will have occurred

slightly earlier than this and, in fact, ruptured follicles were found in sections of the ovaries from some of those rats killed 12 hr. after the injection of UP31.

The occurrence of ova in the tubes was determined in this particular experiment by examination of the tubes in the fresh condition. Since the ova were expressed from the tubes soon after ovulation they were still surrounded by dense masses of cumulus cells and in some instances they could not be counted accurately. They were obviously very numerous.

DISCUSSION

The experiments described above all support the widely held view that ovulation, in the rat at least, is caused by an increase in concentration of luteinizing hormone in the circulation at a particular phase of follicular growth. The condition of the ovaries that are to respond to the gonadotrophic stimulus is, however, as important as the proper secretion of gonadotrophin from the pituitary gland if ovulation is to occur. By using hypophysectomized rats we have been able to study the requisite ovarian conditions. There is now little doubt that the failure to produce ovulation by a single injection of serum gonadotrophin in these rats [Rowlands & Williams, 1941] is due to the atrophy of the ovary. This has now been shown in two ways. If the ovarian atrophy is overcome and the follicles are stimulated by a comparatively small dose of serum gonadotrophin, ovulation is produced by a second and larger injection of serum gonadotrophin (Table 2). The fact that this second dose has to be much larger is in keeping with the relatively low luteinizing potency of this gonadotrophin; the dose given, however, is only a fraction of that we have previously given singly to hypophysectomized rats without producing ovulation. This result also shows that excess follicle-stimulating activity will not inhibit ovulation or luteinization if the luteinizing substance injected with it is acting on follicles that are in a condition for ovulation to occur. Again, if the ovarian atrophy following hypophysectomy is prevented by implantation of tablets of stilboestrol [Williams, 1940] a single injection of serum gonadotrophin will then produce ovulation nearly as readily as it will in an intact rat [Williams, 1943]. The production of ovulation with other extracts, and particularly with chorionic gonadotrophin, accords with their known luteinizing activity. The failure with ox pituitary extract is probably due to inadequate dosage.

The shortness of the phase of follicular growth during which a luteinizing stimulus is optimally effective is shown by the data given in Table 3. If the injection of chorionic gonadotrophin is given simultaneously with the serum gonadotrophin no luteinization occurs;* if it is given 1–3 days after the injection of serum gonadotrophin a premature luteinization of the membrana granulosa occurs which prevents the escape of the ovum. When the injection is delayed longer than 4 days the percentage number of rats ovulating is decreased. This has been attributed to the occurrence of degenerative changes in the ovarian follicles. For the most efficient use of these extracts in the production of ovulation the interval elapsing between their injections is of the utmost importance. It must be remembered that serum gonadotrophin is long-acting since it is equally effective if given as one single injection or as five daily injections; chorionic gonadotrophin on the other hand is quickly destroyed or excreted—50% disappears in the first hour after its injection into intact rats [Zondek, 1940; Zondek,

* An unpublished experiment.

Sulman & Sklow, 1941]. The fact that ovulation occurs 12–14 hr. after the injection of chorionic gonadotrophin (Table 4) indicates its rapidity of action. The fact that two spaced injections of serum gonadotrophin will produce ovulation where one larger injection will not supports the view that the luteinizing and follicle-stimulating factors in serum gonadotrophin are separate entities.

The actual time elapsing between the injection of chorionic gonadotrophin and ovulation is surprisingly constant. This fact will be of practical importance in studies on the hormonal conditions necessary for the maintenance of pregnancy, which we hope to undertake in hypophysectomized rats.

SUMMARY

1. In hypophysectomized immature rats, if the ovarian atrophy is overcome and the follicular system stimulated by an injection of serum gonadotrophin, ovulation can be produced by an injection of chorionic gonadotrophin, pig pituitary gonadotrophin, sheep pituitary gonadotrophin or mares' serum gonadotrophin.

2. The optimal interval between the injections of serum and chorionic gonadotrophins is 4 days.

3. Under these conditions ruptured follicles are found in the ovaries 12–13 hr. after the injection of chorionic gonadotrophin, and ova appear in the tubes 1 hr. later.

4. All ova have passed through the Fallopian tubes, or have disintegrated 96 hr. after the ovulation-producing injection; their behaviour is thus like that of the ova normally produced in the intact rat.

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THE URINARY EXCRETION OF OESTROGENS FOLLOWING THE INJECTION OF PRO-OESTROGENS IN THE GUINEA-PIG

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In previous communications it has been shown that pro-oestrogens differ in behaviour from oestrogens in two ways. First, the dose of a pro-oestrogen which must be placed directly into the vaginae of spayed mice in order to produce the vaginal changes characteristic of oestrus is as large as that needed subcutaneously [Emmens, 1941, 1942*a*]. With oestrogens, the intravaginal dose is from 1/50th to 1/2000th of the subcutaneous dose (i.e. the systemic/local ratio (S/L ratio) is 50 or more). Secondly, a minimal effective dose of a pro-oestrogen, when placed in one of the vaginae of mice possessing two separate vaginal sacs, causes cornification in the second, untreated sac, as well as in the first (Emmens, 1942*b*). Such a dose of an oestrogen, and even much larger doses, do not affect the untreated vaginal sac.

These observations are explicable on the supposition that pro-oestrogens are themselves inert, but that they are wholly or partly metabolized to oestrogens in the tissues, but not in the vagina. The final test of this hypothesis is clearly the demonstration of such a change *in vivo*. Since injected oestrogens or their metabolites are excreted in the urine of laboratory animals [Kunde, D'Amour, Carlson & Gustavson, 1930; Zondek, 1934], it seemed plausible to suppose that, if an oestrogen is formed in the body after the injection of a pro-oestrogen, some of this oestrogen should, at any rate in favourable circumstances, be found in the urine. The detection and estimation of oestrogens formed in this way was therefore attempted.

PROCEDURE

It was found in preliminary tests that guinea-pigs excrete in the urine, in a short period after injection, up to 15 % of an injected oestrogen. Rats, on the other hand, excreted only 0·5 % or less in a similar time. The guinea-pig was therefore chosen as the test animal, and injections were given once daily intraperitoneally in nut oil, for 3 days. The urine was collected for 5 days, covering the period of injection and the 2 days following. The intraperitoneal route was chosen in order to avoid any leakage of the active substance from the site of injection, and its consequent spurious appearance in the urine. The period of collection was limited to 5 days for the sake of speed, since much preliminary testing and adjusting of dosages were necessary. Both male and female animals were used, weighing 250–350 g.

The urine was removed daily from the collecting pot of the metabolism cage and stored without preservative during the period of collection at 25° F. It was then brought to *p*H 1 with concentrated hydrochloric acid. Hydrolysis and extraction with benzene followed according to method 1 of Callow, Callow, Emmens & Stroud [1939]. The benzene extract was evaporated to dryness and the residue taken up in

absolute alcohol for the preparation of solutions in nut oil for tests by the subcutaneous route, or in 50% water-glycerol for tests by the intravaginal route.

The oestrogenic potency of extracts was estimated with spayed albino mice by the methods of Emmens [1939, 1941] for subcutaneous and intravaginal assays respectively, except that control groups of mice receiving international standard oestrone were not always included. From 20 to 60 mice per substance were used in each type of test, and the standard error of estimate of the effective doses (see below) is within 20%, time-to-time variation in sensitivity excepted. However, since comparisons for the computation of ratios were made, as far as possible, from tests conducted at the same time, this source of variation should not be considerable.

Tests with mice possessing two separate vaginae were made as described by Emmens [1942*b*], injection being into the upper vaginal sac.

RESULTS

The potencies of substances assayed in these tests are expressed in terms of effective doses (e.d.), an effective dose being that dose required to produce 50% of positive responses in a group of spayed mice by the particular technique followed. This type of notation is preferable to that of the international unit for the specific purposes of the investigation, as it facilitates the presentation of results, and by its use, the peculiar properties of pro-oestrogens are emphasized.

Oestrogens

Table 1 lists the results with various oestrogens. The number of effective doses injected into the guinea-pigs is always considerably greater as measured intravaginally than as measured by the subcutaneous route.

The urine of control animals receiving no injections, or injections of oil alone, contained 90–600 e.d. per 5 days (mean 377 ± 80 e.d.), by intravaginal assay, and *no* oestrogen by subcutaneous assay. The urine of two males showed the same titre as females.

Intravaginal assays of all urines collected from animals injected with oestrogens other than oestradiol indicated a lower percentage excretion of the oestrogen or its metabolites than assays by the subcutaneous route. To put the statement another way, the S/L ratio of the urine extracts was lower than that of the injected oestrogens. The urine from the animal receiving oestradiol, however, gave a higher S/L ratio (200 as against 50 for pure oestradiol). Since oestradiol is probably excreted as oestrone and/or oestriol [Pincus & Zahl, 1937] this is not remarkable, as the S/L ratio of both of these substances is higher than 200. Diethylstilboestrol is excreted as such, or in esterified form [Mazur & Schorr, 1942], and so would be present in extracts as the free compound. So, probably, would α-ethyl-β-phenyl-stilboestrol, whereas oestrone might in part be converted to oestriol prior to excretion [Pincus & Zahl, 1937].

There thus appears to have been a potentiation of the oestrogens in extracts when assayed in oily solution subcutaneously or an inhibition of the same substances when assayed in water-glycerol intravaginally. The latter is unlikely, as even esterification does not much alter the activity of a compound when tested locally [Emmens, 1941], whereas the former is very likely, as the extracts contained benzoic acid and other inactive material likely to potentiate the oestrogens concerned, and similar extracts

Table 1. *The urinary excretion of oestrogens by guinea-pigs after the intraperitoneal injection of oestrogens in nut oil*

Compound injected	No. and sex of pigs	Amount mg.	No. of effective doses approx.	S/L ratio of injected compound	Amount recovered in effective doses	% Recovery	Method of assay	S/L ratio of urine extract
Nil	7 ♀	—	—	—	377*	—	I.V.†	—
	2 ♂	—	—	—	470	—	I.V.	—
Oestrone	1 ♀	0·3	1,000,000	250	25,000	2·5	I.V.	47
			4,000		553	13·8	S.C.‡	
	1 ♀	3·0	10,000,000	250	313,000	3·1	I.V.	52
			40,000		6,000	15·0	S.C.	
	1 ♂	3·0	10,000,000	250	250,000	2·5	I.V.	45
			40,000		5,800	14·5	S.C.	
Oestradiol	1 ♀	3·0	6,000,000	50	500,000	8·3	I.V.	200
			120,000		2,700	2·1	S.C.	
Diethyl-stilboestrol	1 ♀	0·03	75,000	320	4,000	5·2	I.V.	110
			250		33	13·2	S.C.	
	1 ♀	0·3	750,000	320	62,500	8·3	I.V.	150
			2,500		333	13·2	S.C.	
	1 ♀	0·6	1,500,000	320	50,000	3·3	I.V.	200
			5,000		250	5·0	S.C.	
	1 ♂	0·6	1,500,000	320	42,500	2·8	I.V.	120
			5,000		375	7·5	S.C.	
	1 ♀	3·0	7,500,000	320	425,000	5·6	I.V.	260
			25,000		2,000	8·0	S.C.	
α-Ethyl-β-phenyl-stilboestrol	1 ♂	6·0	9,000,000	1400	68,000	0·8	I.V.	380
			6,600		166	2·5	S.C.	

* 377 ± 80 (σ_m). † Intravaginal. ‡ Subcutaneous.

of human urine are known to do so [Emmens, 1939]. We may conclude, therefore, that the intravaginal assays give more correct figures for the content of active substances in such extracts—given that their chemical nature is known. By either technique the percentage excretion appears to be unaffected by the amount of material injected (Table 1, col. 7).

Pro-oestrogens

Table 2 lists the pro-oestrogens tested. They were those of which sufficient material was available for relatively large-scale tests, and thus comprise in effect a random sample. The e.d. of a pro-oestrogen is unaffected, or practically so, by the route of administration and the number of e.d.'s injected remains the same, whichever method of assay is considered. Thus, the e.d.'s of the three compounds giving measurable excretion in the urine are, for the intravaginal and subcutaneous routes respectively: α-phenyl-stilboestrol, 10 and 15μg.; α-phenyl-β:β-di(*p*-hydroxyphenyl)-ethylene, 8 and 10μg. (revised assays); 9:10-dihydroxy-9:10-dihydro-9:10-di-*n*-propyl-1:2:5:6-dibenzanthracene, 18 and 20μg. The mean of these determinations has been used in each case as measuring the e.d.

In the amounts injected, the four substances heading the list in Table 2 did not increase urinary oestrogens. The next three increased them up to 10-fold, a highly significant increase, as measured intravaginally, and more than 10-fold as measured subcutaneously. The additional activity was, moreover, due to the presence of

Table 2. *The urinary excretion of oestrogens by guinea-pigs after the intraperitoneal injection of pro-oestrogens in nut oil*

Compound injected	Sex of pig	Amount mg.	No. of effective doses	Amount recovered in effective doses	% Recovery	Method of assay	S/L ratio of urine extract
Dihydroxy-di-α-naphthyl-acenaphthene	♀	150	500	Nil	—	I.V.*	—
Triphenylethylene	♀	300	1000	Nil	—	I.V.	—
α-(4-Hydroxyphenyl)-stilbene	♀	12	400	Nil	—	I.V.	—
p-Hydroxypropiophenone pinacol	♂	240	5300	18	—	S.C.†	—
α-Phenyl-stilboestrol	♀	12	1000	1200	120	I.V.	—
	♀	60	4800	5500	115	I.V.	—
				<500	<11	S.C.	>11
	♂	60	4800	4000	84	I.V.	17
				250	5	S.C.	
α-Phenyl-β:β-di(*p*-hydroxy-phenyl)-ethylene	♀	60	6600	3100	47	I.V.	12
				280	4	S.C.	
	♂	60	6600	5000	76	I.V.	45
				110	1·6	S.C.	
9:10-Dihydroxy-9:10-dihydro-9:10-di-*n*-propyl-1:2:5:6-di-benzanthracene	♂	120	6000	1300	22	I.V.	72
				18	0·3	S.C.	

* Intravaginal. † Subcutaneous.

oestrogens, with perhaps only a small amount of pro-oestrogens, since the S/L ratios of the urine extracts were in all cases high, although not as high in general as those of the urine extracts listed in Table 1. Following the administration of α-phenyl-stilboestrol, moreover, the total oestrogenic potency of the urine as measured intravaginally was somewhat in excess of that of the oestrogen injected. We cannot therefore suppose that the data here presented can be explained on any hypothesis involving the supposition that α-phenyl-stilboestrol is excreted unchanged, the more so since the period over which the urine was collected was probably too short to allow of complete excretion, nor can the method of extraction be guaranteed to have 100% efficiency.

Table 3. *The urinary excretion of oestrogens after the injection of pro-oestrogens in guinea-pigs as shown by the response of mice with two vaginal sacs*

Substance	No. of effective doses per mouse	No. of mice	No. of positives in Upper vaginae	No. of positives in Lower vaginae	S/L ratio in usual test
α-Phenyl-stilboestrol	1	5	1	1	
	2	9	3	2	1·5
	7	10	8	8	
Urine extract after injection of α-phenyl-stilboestrol	1	9	6	0	>11·0
	4	30	22	0	
Urine extract after injection of α-phenyl-β:β-di(*p*-hydroxyphenyl)-ethylene (isomer of above)	20	18	18	3	12·0

In Table 3, confirmatory data as to the oestrogenic nature of the substances in the extracts are presented. The response of mice with two vaginae to α-phenyl-stilboestrol is contrasted with that to the extract of the urine from animals receiving the compound, and its isomer, α-phenyl-β:β-di(*p*-hydroxyphenyl)-ethylene. Whereas the pro-oestrogen, as already reported [Emmens, 1942*b*], causes reactions in both vaginal sacs when placed only in the upper vagina, it required 20 times the effective dose of the urinary extract to produce a few responses in the lower vaginae of a group of mice. This reaction is typical of an oestrogen but with probably a little pro-oestrogen mixed with it, since a pure oestrogen would normally fail to elicit responses in the lower vaginae at a level even of 20 e.d.

DISCUSSION

The excretion of oestrogenic material in guinea-pig urine is demonstrable when oestrogens are injected into the animal, and, within the small range of compounds used, the percentage excretion does not vary a lot from substance to substance or from dose to dose of the same substance (although α-ethyl-β-phenyl-stilboestrol gave rather low figures). The excretion following the administration of pro-oestrogens is much less predictable.

However, when such excretion occurs, it is predominantly of an oestrogen, and may in some cases be entirely so. It is to be expected that some of the injected material will pass into the urine. Indeed, on the basis of the excretion of diethylstilboestrol and α-ethyl-β-phenyl-stilboestrol, it might reasonably be supposed that up to 10% of the closely allied α-phenyl-stilboestrol would be similarly excreted. The figures in Table 2 relevant to assays by the subcutaneous route suggest that this is what occurs, but when assays made by the intravaginal method are examined it is clear that only a fraction of the activity then found can be due to the passage of the injected material into the urine. Any pro-oestrogen passing unchanged into the urine will contribute the same number of e.d.'s to the oestrogenic titre of the extract, whether it is assayed by the subcutaneous or by the intravaginal route. The finding of a large excess of effective intravaginal doses as compared with effective subcutaneous doses means that an oestrogen is present. Thus, to take an example from Table 2, the urine of the male guinea-pig injected with α-phenyl-stilboestrol gave an extract containing 250 effective subcutaneous doses, and 4000 effective intravaginal doses. On the assumption that the oestrogen concerned has, in urine extracts, an S/L ratio of 100, and that the S/L ratio of the pro-oestrogen remains unity, we find that the extract must contain approximately 210 e.d.'s of pro-oestrogen, however it is assayed, and 38 effective subcutaneous doses of oestrogen, equivalent to 3800 effective intravaginal doses. By the intravaginal route one is, therefore, measuring practically only the content of oestrogen in the extract, not of pro-oestrogen. With the dibenzanthracene compound (Table 2) it is not necessary to suppose that any of the parent substance passes into the urine unchanged, as the S/L ratio of 72 is high enough to be typical of a pure oestrogen.

There is no obvious correlation between chemical structure and the behaviour of a pro-oestrogen in these tests. α-(4-Hydroxy-phenyl)-stilbene, triphenylethylene, *p*-hydroxypropiophenone pinacol, α-phenyl-stilboestrol and α-phenyl-β:β-di(*p*-hydroxyphenyl)-ethylene are related chemically, yet only the last two caused a rise

of urinary oestrogens. The dibenzanthracene compound, however, remote in structure from any known oestrogen, also caused a measurable rise, and the urine from the animal receiving it had the highest S/L ratio of the series. It has not been possible to test the properties of tri(*p*-hydroxyphenyl)-ethylene. This compound, if it proved to be an oestrogen, would seem to be the most likely metabolite to be formed from these di-phenols, but there is no reason to suppose that it is an oestrogen; from its structure it is quite probably a pro-oestrogen. It seems idle to speculate as to what substance may be formed from the dibenzanthracene compound, without chemical studies of the urine contents.

The fact that no excretion of oestrogenic material occurs after the injection of some pro-oestrogens is, of course, further evidence against any supposition that their action can depend on the production of endogenous natural oestrogen. If this were so, a proportion of the endogenous oestrogen would, as in the case of oestrone and oestradiol, be excreted in the urine. Thus, 5000 e.d.'s of *p*-hydroxypropiophenone pinacol must, on the endogenous oestrogen hypothesis, produce 5000 (subcutaneous, or the equivalent of subcutaneous) doses of, say, oestradiol. Of these, a detectable proportion would certainly pass into the urine. This argument applies even to the compounds producing a rise in urinary oestrogens, for the level of excretion, as measured intravaginally, although high relative to the intravaginal potency of the injected compound, is very low for subcutaneously equivalent amounts of oestrone or oestradiol. Thus, 4800 effective doses of α-phenyl-stilboestrol stimulated the excretion of 5500 intravaginal doses of an oestrogen. The approximately equivalent 4000 subcutaneous doses of oestrone in Table I stimulated the excretion of 25,000 intravaginal doses.

SUMMARY

1. The S/L ratio of oestrogens in the urine of guinea-pigs injected with the oestrogens oestrone, diethylstilboestrol and α-ethyl-β-phenyl-stilboestrol is high, but lower than that of the injected compounds. The fall in the ratio is probably due to the potentiation of the urinary oestrogens by other inert substances in the extracts, when assayed by subcutaneous injection in oily solution. The S/L ratio of the urine of an animal receiving oestradiol was higher than that of oestradiol, but lower than that of the probable excretion products, oestrone and oestriol.

2. The S/L ratio of oestrogens in the urine of guinea-pigs injected with certain pro-oestrogens is also high, but in most cases still lower than the ratios found after injection of oestrogens. The high ratio (17 to 72) shows that the excreted oestrogenic compounds are, in fact, true oestrogens, but its relative fall indicates that in some cases at least a certain amount of the injected material passes into the urine unchanged.

The oestrogenic nature of the excreted products is further demonstrated by their behaviour in tests with mice possessing two separate vaginal sacs, since injection into one sac of any but a high multiple of the effective dose did not affect the second sac.

3. After the injection of other pro-oestrogens, no increase in urinary oestrogen was found, and this in itself is evidence that their activity is not due to stimulation of the production of endogenous natural oestrogens, for a detectable percentage of these would be excreted.

4. It has thus been possible to demonstrate that, when an increase in the titre of urinary oestrogenic activity followed the injection of pro-oestrogens, this increase was

due predominantly or entirely to the presence of oestrogens. The hypothesis that pro-oestrogens are converted to oestrogens in the body has thus been verified.

I am again indebted to Prof. E. C. Dodds, F.R.S. and Mr W. Lawson for the generous provision of most of the synthetic compounds used.

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ON STAINING THE BASOPHIL CELLS OF THE HUMAN HYPOPHYSIS WITH SPECIAL REFERENCE TO THE ABNORMAL BASOPHIL CELLS OF CUSHING'S SYNDROME

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Pathological abnormality in the basophil cells of the human hypophysis in cases of Cushing's syndrome may be very complex. A single basophil cell may show areas of normal granularity, areas of refractile hyaline cytoplasm (of Crooke), normal granules dispersed around vacuoles, conglomerations of small vacuoles outlined by delicate refractile cytoplasmic threads, one or more nuclei pressed out as a fine rind and scalloped by the vacuoles, and cytoplasmic processes extending out and embracing surrounding cells [McLetchie, 1944]. Though it may be that all this complexity is easily analysed in fresh material, this is not so in material 12–30 hr. *post mortem*, with which I, and most morbid anatomists, have to deal.

I have found that if a stain is specific for β (basophil) granules then the hyaline cytoplasm of Crooke will appear unstained. While under certain conditions many dyes (iron and chrome haematoxylins, acid fuchsin, aniline blue, methyl blue, crystal violet, ethyl violet) will stain β granules specifically, I have found it necessary in this study to demand that the method must permit not only of tinctorial differentiation of granules from hyaline material but also of differential nuclear staining. The methods described here give brilliant tinctorial distinctions between the components, nuclear, granular and cytoplasmic, of the abnormal basophil cells of Cushing's syndrome. Acid fuchsin is used as the main vehicle of distinction for these elements, even when depicting the basophil cells finally in their traditional colouring with aniline blue. Methods have been introduced for clarity of differentiation to eliminate, alter, or reduce the affinity of the acidophil (α) granules for acid fuchsin.

MATERIALS

Nuclear staining with haemalum. Overstain with haemalum (Mayer's or Harris's), differentiate with acid alcohol. Any common haematoxylin nuclear stain may be used, but should be differentiated as β granules take up some nuclear stains.

Acid fuchsin. A 2% aqueous solution of acid fuchsin containing 1% of glacial acetic acid.

Aniline blue. A 2% aqueous solution of aniline blue (soluble blue, C.I. 706 or 707), containing 1% of glacial acetic acid.

Pyrrol blue. Where this blue is mentioned after aniline blue it refers to the use of this dye, in $\frac{1}{2}$% aqueous solution, as being preferable to aniline blue. Pyrrol blue (soluble blue, C.I. 710—Vector) overlays acid-fuchsin-stained β granules at a slower

rate than aniline blue. If pyrrol blue is not obtainable, a ½% aqueous solution of aniline blue should be used.

Alcoholic phosphotungstic acid. 95% ethyl alcohol, containing 2% of phosphotungstic (or phosphomolybdic) acid. The slide should be rinsed in 95% ethyl alcohol before use.

Strong differentiator. Phosphotungstic acid, 2 g.; saturated solution of picric acid in 95% ethyl alcohol, 70 ml.; water, 30 ml.

Weak differentiator. Phosphotungstic acid, 2 g.; saturated solution of picric acid in 95% ethyl alcohol, 40 ml.; water, 60 ml.

Picro-orange. A 0·2% solution of orange G (C.I. 27) in 80% ethyl alcohol, saturated with picric acid [Lendrum & McFarlane, 1940].

Crystal violet. A 0·5% aqueous solution of crystal violet.

Aniline oil/xylol. Aniline oil, 1 part; pure xylol, 4 parts.

FIXATION

The methods to be described work with all common Zenker and formalin fixatives. I have usually employed, and find preferable, the following methods.

(1) Fix the whole gland in 10% formalin or Formol-Zenker (equal parts of 10% formalin and Zenker's solution without acetic acid) for 1 hr.

(2) Bisect in the horizontal plane, fix in Formol-Zenker for 5–6 hr.

(3) Transfer gland to saturated aqueous solution of mercuric chloride for 48–72 hr.

Dehydration is carried out in a butyl alcohol series [see Muir & Ritchie, 1937]; clearing in butyl alcohol/chloroform—chloroform series; embedding in paraffin for 24 hr. If the gland proves to be of exceptional interest, the two blocks are bisected in the vertical plane and re-embedded; the smaller blocks are more suitable for cutting thin sections. If 10% formalin is used alone, fixation should be continued for 72 hr. and the sections require mordanting in Muller's fluid (24 hr.) followed by washing in tap water (10 min.).

In my experience, basophil-cell hyalinization is so conspicuous in the hypophysis from cases of Cushing's syndrome that visual inspection of the hyaline cytoplasm and β granules, at various stages of differentiation in the staining methods, can be carried out with the low power of the microscope.

STAINING METHODS

Method I. *A specific acid-fuchsin stain for β granules, with differentiation of hyaline cytoplasm*

Principle

If a section is mordanted in alcoholic phosphotungstic acid and subsequently stained with acid fuchsin, only the β granules and stroma are stained. All other elements, including α granules and hyaline cytoplasm, remain uncoloured. On staining with aniline blue, the connective tissue and hyaline cytoplasm rapidly become blue. Thus, in the hyaline basophil cell a brilliant contrast is obtained between granules (red) and hyaline (blue) material, while inspection of these elements is made easy since the cytoplasm of acidophil and chromophobe cells appears practically unstained.

Technique

(1) Mordant section in alcoholic phosphotungstic acid for 2–5 min.

(2) Rinse briefly in tap water.

(3) Stain in acid fuchsin, inspecting at intervals. In a short time (2–6 min.) the β granules and stroma become bright red; no other elements in the anterior pituitary are stained, apart from a faint red staining of the nuclei. In hyaline basophil cells the hyaline cytoplasm remains unstained and appears as clear refractile zones contrasting with the brightly stained β granules. Stop staining with acid fuchsin when the β granules are bright red, but still as discrete as possible; rinse briefly in tap water. (Overstaining can be reduced by washing in tap water.)

(4) Stain in aniline blue (pyrrol blue), inspecting at 1-min. intervals. The stroma rapidly takes on blue, later (2–5 min.) the hyaline cytoplasm becomes stained a deep blue. Stop staining when maximum contrast is obtained between hyaline cytoplasm (blue) and β granules (red).

(5) Rinse in 1 % aqueous acetic acid; dehydrate rapidly and mount.

Result

Basophil cells: β granules, bright red; hyaline cytoplasm, blue; nuclei, faint red. Cytoplasm of the acidophil and chromophobe cells, very faint diffuse blue or red; acidophil-cell nuclei take on the aniline blue if the duration of the staining is sufficient (Plate 2, fig. 3).

Modifications of method I

Nuclear reinforcement. Sharp nuclear detail (dark brown) is obtained by staining with haemalum, with or without blueing. The nuclear staining is introduced before the method described.

Iodine. Iodine has a very powerful mordanting effect in increasing the acid-fuchsin specificity of β granules. It is used in the above method by mordanting the section in Gram's iodine for 2–5 min. before mordanting in alcoholic phosphotungstic acid. The iodine coloration is rapidly removed from the section in the latter solution. Iodine cannot be used where deep nuclear staining is required.

Notes

If aniline blue staining is continued beyond the limits stated, the acid-fuchsin-stained β granules are eventually overlaid with aniline blue to become almost black; the granules of immature cells (feebly stained) are overlaid before the granules of mature cells. The tinctorial contrast between β granules and hyaline cytoplasm in terms of blue can be increased, and staining of other elements eliminated, by subsequent treatment with the weak differentiator.

METHOD II. *Differential staining of all the elements in the anterior pituitary with special reference to the pituitary gland of Cushing's syndrome*

This method is described because it allows depiction of the anterior pituitary in terms of Mallory's original method. It is essentially a modification of the Mallory technique.

Principles

Picration. Lendrum & McFarlane [1940] have shown that the introduction of an alcoholic solution of picric acid containing orange G would ensure a uniform yellow coloration of the red blood cells when using Mallory's stain, no matter what fixative was used. This solution also prevents the full staining of the acidophil cells with acid fuchsin. The degree of staining of the acidophil cells may be controlled by adjustment of the duration of immersion in picro-orange and acid fuchsin and of the intermediate washing in tap water (cf. Table 1). If it is desired to have the α granules stained red

Table 1

	Staining procedure (min.)			Results		
	Picro-orange	Washing in tap water	Acid fuchsin	R.B.C.	α granules	β granules
1	2	20	10	Yellow	Red	Red
2	2	7	10	Yellow	Deep orange-red	Red
3	2	5	10	Yellow	Light orange	Red
4	10	1	5	Yellow	Yellow	Red

rather than orange in subsequent stages then it is only necessary to stain for a longer time in acid fuchsin; the picric acid is dissolved out of the cells into the staining solution and further acid-fuchsin staining occurs. The practical advantages of picration are: the yellow coloration of the red blood cells; facilitation of the control of subsequent acid-fuchsin staining since the orange-red acidophil cells are easily differentiated from the deep red basophil cells; the acidophil cells can be depicted orange or orange-red when acid-fuchsin staining is used for β granules.

Differentiation. In sections stained with acid fuchsin all elements, including α and β granules, are stained and removal of the stain from the different elements by alcoholic phosphotungstic acid is insufficiently controllable to be of practical use. Decolorization of the stroma and hyaline cytoplasm may be obtained by using aqueous phosphomolybdic acid or better by using alcoholic phosphotungstic acid containing picric acid (strong differentiator). When Formol-Zenker/corrosive sublimate-fixed sections are stained with the standard acid fuchsin, subsequent washing in tap water decolorizes the β granules but not the α granules. If acid fuchsin containing 10% of acetic acid is used the conditions are reversed, the β granules being strongly stained and the α granules only feebly; this may account for the action of the picro-orange described above since the latter solution is acid. Standard acid fuchsin containing 1% of acetic acid also shows little distinction between β granules and hyaline cytoplasm, whereas the solution with the higher concentration of acetic acid only allows feeble staining of the hyaline material. This may explain the advantages of using acid-containing differentiators rather than aqueous phosphomolybdic acid solution, though the alcohol content of the weak and strong differentiators used here may be partly responsible for the better action (cf. method I, where mordanting with alcoholic phosphotungstic acid confined the acid-fuchsin staining to the β granules).

Counter-staining. Two methods are available when β granules are stained with acid fuchsin and stroma and hyaline cytoplasm decolorized; either subsequent aniline-blue staining is stopped when the stroma and hyaline cytoplasm are stained (method II A), or the aniline-blue staining is continued until the acid-fuchsin-stained

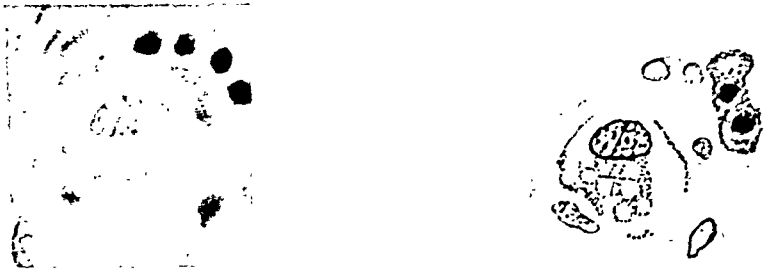


FIG. 1. A hyaline basophil cell with hyaline cytoplasm stained blue (aniline blue) and β granules stained red (acid fuchsin) while the acidophil cells appear orange (acid fuchsin partially suppressed by picro-orange).
There is vacuolation of the juxta-nuclear granules in the hyaline cell. ×800. Modified Mallory, method IIA.

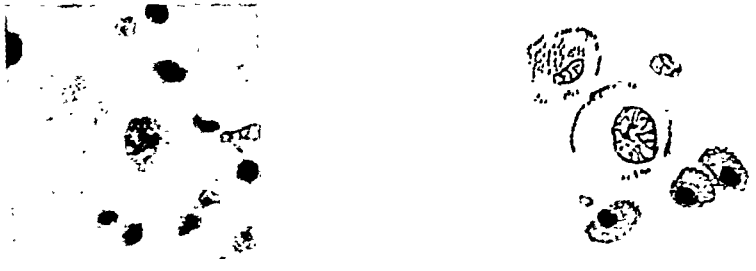


FIG. 2. Two hyaline basophil cells with β granules stained dark blue (acid fuchsin overlaid with aniline blue) and hyaline cytoplasm stained grey (excess aniline blue removed by weak differentiator) while the acidophil cells are stained red (acid fuchsin without suppression by picro-orange).
The two small cells are chromophobe cells. ×800. Modified Mallory, method IIB.

All sections from cases of Cushing's syndrome, 12–26 hours *post mortem*.

described it because it is only by going through the stage of differentiation necessary in method II A that the fine distinction between β granules and hyaline cytoplasm can be obtained in method II B. I consider that a criterion of differentiation similar to that which I have described will be required at some stage of differentiation before any trichromic staining method will give good differentiation of the various elements in the abnormal basophil cells of Cushing's syndrome. Method II B gives the tinctorial distinctions between granules and nuclei on which Severinghaus bases his chromophobe/chromophil pituitary cycles [Severinghaus, 1938]. In staining normal glands, I use the following criteria of differentiation:

Stage (3), stop staining with acid fuchsin when β granules are as deeply stained as possible without allowing acid-fuchsin staining of the red blood corpuscles.

Stage (4), differentiate for 1 min.

Stage (7), differentiate until β granules are as discrete as possible.

METHOD III. *The modified Gram's stain for β granules of the anterior pituitary*

Principle

When using Gram's stain for bacteria, the basophil granules are strongly Gram-positive relative to all other structures in the anterior pituitary.

Technique

(1) Section to water.
(2) Stain with crystal violet, 5 min.
(3) Mordant in 10 % aqueous solution of sodium chloride, 10 min.
(4) Mordant in Gram's iodine, 10 min.
(5) Rinse in water.
(6) Blot dry.
(7) Differentiate in aniline oil/xylol, rinsing in pure xylol and inspecting at intervals. All the elements except the β granules are rapidly decolorized (1–3 min.), leaving the β granules deep blue, almost black, as the only stained elements. The reaction is highly specific and it may be as long as 20 min. after the other elements are decolorized before excess crystal violet is removed sufficiently from the β granules for them to appear discrete. This latter decolorization, if too slow, should be hastened by using a greater proportion of aniline oil in the xylol or by breathing on the slide. Saline was introduced for Gram's stain by Kirkpatrick [see Muir & Ritchie, 1937]. It is unnecessary to use saline in the staining of fresh glands, but I have found that its use makes the reaction more specific in older material. In fresh material (4–6 hr. *post mortem*) differentiation can also be carried out by acetone-xylol mixtures. The Gram reaction is so highly specific that I have never failed to stain β granules discretely in material up to 24 hr. *post mortem*.

In this method, hyaline cytoplasm is very rapidly decolorized (1 min.) and stands out as granule-free refractile areas, contrasting with the heavily stained β granules. As a counterstain, I usually employ neutral red (10 min.), followed by carmalum (10 min.). Counterstaining is introduced before the general method. With this method, hyaline cytoplasm is coloured reddish yellow, contrasting with the red nucleus and the almost black β granules, while all other granular elements in the anterior pituitary are only feebly stained with the counterstain.

HISTOLOGICAL FINDINGS

Apart from the easily obtained brilliant contrast which the specific acid-fuchsin stain and the modified Gram's stain make between β granules and hyaline, I have found these methods of value in the study of immature cells. Anyone who studies adult human anterior pituitary glands from routine autopsy material must be struck with the great variation of tinctorial affinity and granular distribution in cells which are clearly neither mature chromophil nor chromophobe cells. Using any modified Mallory or eosin/methylene blue stain on post-mortem material, I have found that the granules of many of these immature cells can be made to take on characters of the granules of either the acidophil or basophil line. While this is largely due to the insurmountable difficulty of post-mortem change and, in any case, care should naturally be taken in making any deduction from the appearances, I believe that the specific acid-fuchsin stain for β granules and the modified Gram stain are so highly specific that any immature cell which shows, by these methods, some granules stained similarly to the granules of the mature basophil cells belongs to the basophil line. In addition, the staining of the granules with one dye alone obviates the complexity of having the granules stained by a combination of dyes which, as I have already pointed out, may obtain in modifications of the Mallory method.

Some authors have referred to Crooke's hyaline change as a process of degranulation. In addition to having the very different staining properties from β granules, which we have described, the hyaline cytoplasm of Crooke is refractile, granule-free, and has a regular mode of distribution in the cell [Crooke, 1935]. The earliest lesion appears as a minute subperipheral zone a few granules below the surface [see McLetchie, 1944, Fig. 5]; thence hyaline material advances centripetally, leaving in more advanced hyalinization two granular areas: one on that side of the eccentric nucleus having the most abundant cytoplasm, and another on the periphery of the cell. Ultimately, the cell may be completely hyalinized, though more commonly remnants of the peripheral granular rim persist. On the other hand, the term 'degranulation' has long been applied to a process in the basophil cells which is present to some extent in every human hypophysis. The basophil cells contain areas which are not entirely granule-free but have a sparser distribution of granules than the surrounding cytoplasm. The granules in the area lie in a ground substance which is not refractile but has the almost imperceptible fine matt surface similar in appearance to the chromophobe cytoplasm in post-mortem material, and which has the indifferent staining properties of chromophobe cytoplasm as opposed to the brilliant coloration which hyaline cytoplasm can take on. Furthermore, these areas of degranulation have no systematic mode of distribution in the cell like that of hyaline cytoplasm, though they are, in my experience, found most commonly in a juxta-nuclear position, which is the opposite to that of the distribution of hyaline. It is obvious that basophil hyalinization should be clearly distinguished from the physiological process of basophil-cell degranulation. Severinghaus [1938] has correlated the processes of degranulation in the chromophil cells of the normal hypophysis with nuclear changes and, on the appearances, has based his theory of chromophil/chromophobe cycles.

Large chromophobe cells are sometimes present in the anterior pituitary which, in fresh material, can be shown to be predestined basophil cells. These cells are large, of

indefinite outline, and send processes between surrounding cells. They have a large vesicular nucleus. They show no β granules with specific stains (methods I and III) for β granules. But, in method I, on introducing aniline blue and removing excess dye with the strong differentiator, they exhibit a delicate spongioplasm which stains with aniline blue at a stage when the acid-fuchsin-stained β granules are still unaffected by the blue and the acidophil and chromophobe cells are decolorized. This same spongioplasm may be seen in cells of slightly greater maturity in which a few granules are present which stain faintly with specific stains for β granules. Hence, I consider that this spongioplasm represents a stage preceding the development of β granules in immature basophil cells. In some cases I have recognized a process of agglutination and shrinkage of the spongioplasm so that it stains more intensely with aniline blue, and finally, when this process is complete, the nucleus becomes pyknotic and the cytoplasm has usually numerous perforations. Though the cytoplasm will now stain a deep blue, it does not give any of the specific reactions of basophil granules. We consider that this cell is the same as the 'agglutinated' basophil cell described and illustrated by Crooke & Russel [1935], which is often prominent in the hypophysis in Addison's disease. Thus, we consider that the 'agglutinated' basophil cell represents the end-product of a degeneration of the primitive basophil cell. Stages in the degeneration should not be confused with hyalinization, as the agglutinated spongioplasm has never the refractile waxy character of hyaline cytoplasm, is not associated with a regular mode of distribution in a cell which has ripe β granules, and is ultimately associated with nuclear pyknosis.

I have not had the opportunity of fixing human hypophyses earlier than 4 hr. after death. Our material is usually obtained from 8 to 30 hr. *post mortem*. It is well known that the staining of material fixed immediately after death may present a different problem from staining material fixed some hours later. Some observations I have made may be of benefit to those who wish to apply these methods to very fresh material. The Gram's stain shows increasing specificity with increasing freshness, and I consider that it should work with material fixed immediately after death. The prevention of acid-fuchsin staining of the acidophil cells by picration becomes more potent the fresher the material, and it is more marked with chromate-containing fixatives than with those that do not contain chromate. Hence, when acid-fuchsin staining of acidophil cells is desired, as in method II, picration should be avoided in fresh material as it may take hours to get slight acid-fuchsin staining of acidophil cells even after short exposure to picro-orange. While the acid-fuchsin specificity of β granules after alcoholic phosphotungstic acid mordanting (method I) becomes more marked the fresher the material, in some cases subsequent aniline-blue staining produces a very rapid overlaying of the acid-fuchsin-stained β granules with blue; hence, in making observations on hyaline cytoplasm, a very dilute aniline blue will require to be used.

SUMMARY

Two simple staining techniques are introduced for staining the β granules of the hypophysis specifically and for differentiating the hyaline cytoplasm of Crooke from β granules.

Modifications to obtain maximum contrast between β granules and hyaline cytoplasm in the traditional Mallory picture of the hypophysis are described.

The differences between degranulation and hyalinization in the basophil cells of the hypophysis are described.

I am indebted to Professor J. Shaw Dunn for advice and criticism. The strong and weak differentiators were suggested to me in February 1941 by my colleague, David McFarlane. With slight modifications they form the basis of the routine trichromic stain used in this Department (Picro-Mallory) [D. McFarlane, unpublished]. Grateful acknowledgement is made to the Rankin Medical Research Fund, Glasgow University, for a grant to defray expenses, to Mr Kirkpatrick and Mr Stamp for photomicrography, and especially would I like to thank Dr A. C. Lendrum for early instruction in histological technique and in the preparation of coloured illustrations.

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THE PITUITARY BASOPHILISM SYNDROME OF HARVEY CUSHING

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Since Cushing [1932] described the syndrome of pituitary basophilism (Cushing's syndrome) many cases of the condition have been published. The syndrome is characterized by an obesity sparing the limbs, by marked hypertension, glycosuria and hyperglycaemia, osteoporosis, characteristic cutaneous striae, polycythaemia, amenorrhoea and hypertrichosis in the female, impotence in the male, asthenia and diminished resistance to infection [Cohen & Dible, 1936]. Pathological findings in the condition have been very varied in cases which have been clinically indistinguishable (see Crooke's [1935] series). By 1936 the following abnormalities had been described: basophil adenoma and basophilia (relative increase of pituitary basophil cells); adreno-cortical hyperplasia, adenoma, carcinoma; and thymic carcinoma associated with adreno-cortical hyperplasia. In a few cases neither adreno-cortical, thymic, nor anterior pituitary tumours were recorded [Oppenheimer, Globus, Silver & Shaskin, 1935; Freyberg, Barker, Newburgh & Coller, 1936; Crooke, 1935; Cohen & Dible, 1936].

It seemed remarkable that such diverse pathological findings should be associated with one clinical condition until Crooke [1935] found an abnormality common to all. Crooke investigated the pituitaries of a series of twelve (now fifty—personal communication) cases of Cushing's syndrome, many of which had been reported elsewhere. The series contained examples of all lesions then known to be associated with the syndrome. In every case he found a conspicuous degree of hyalinization of the cytoplasm of the basophil cells of the anterior pituitary; this change was found in only a very few cases (9) of a large non-basophilism series (350), and there only in a partial form in a very few cells; in these latter cases it was not constantly associated with any condition. Crooke concluded that basophil-cell hyalinization was the abnormality of fundamental significance in Cushing's syndrome. Since then a specific nuclear abnormality associated with hyalinization of the basophil cells has been described [Severinghaus, 1938].

In one of three hypophyses from cases of basophilism which I have examined, another complex basophil abnormality in addition to basophil-cell hyalinization is present. The abnormality appears to be the same as that described by Severinghaus, but I place an entirely different interpretation on the appearances. Furthermore, the complex basophil abnormality present has an important bearing on recent experimental work. It is therefore my purpose in this communication to describe the hypophysis of this case in detail. For control purposes a series of hypophyses from conditions other than basophilism has been examined.

DESCRIPTION OF CASE

History. The patient, an unmarried male aged 32, was admitted to the surgical wards of Mr J. S. Buchanan, Western Infirmary, Glasgow, with cellulitis and abscess formation of the left foot caused by slight trauma. The patient had not felt well for the past three years; he had noticed his hair becoming grey and obesity developing over the past two years; he had been worried by a feeling of 'uselessness' and lack of libido for the past eighteen months; for the past year he found that he only had to shave once every three days instead of every day, as previously. There was a clinical history of diabetes mellitus for the past twelve months. Before all this he had been a normal healthy subject.

On admission the patient was grey-haired and of florid complexion. He was of average stature but was very stout, showing especially facial and abdominal obesity. A few bluish striae were present on the abdomen. Glycosuria, polyuria, acetonuria and polydipsia were present. There was hypertension (B.P. 170/100). The heart was enlarged; the subcutaneous and retinal vessels showed arteriosclerotic changes. The blood picture gave R.B.C. 5·1 million, W.B.C. 7400 and Hb 90%. Bone changes were not obvious but the patient was too ill for extensive investigation. The Wassermann reaction was negative. Radiography of the sella turcica showed no abnormality. The condition was diagnosed as Cushing's syndrome.

The patient's condition deteriorated both locally and generally, despite control of the glycosuria by insulin and repeated drainage of the septic area. Ultimately attacks of blindness, giddiness and mental upset became frequent, and the patient died in coma six weeks after admission. Post-mortem examination was carried out by Professor J. Shaw Dunn 26 hr. later.

Summary of post-mortem findings: Marked hypertrophy of left ventricle, arteriosclerosis and bronchopneumonia. Endocrine glands: pancreas embedded in fat but showing a considerable amount of natural-looking tissue; adrenals (12 g.) not enlarged; thyroid not enlarged, no abnormality; testes small but of normal appearance; pituitary gland of normal size; no thymic tissue found in the mediastinum. Only the pituitary gland was taken for histological examination. The paraffin blocks of the gland were given to me by Dr J. E. Craik.

HISTOLOGICAL EXAMINATION

The pituitary gland had been divided into four parts by cuts parallel to the sagittal plane and fixed in Formol-Zenker/corrosive sublimate. The posterior lobe was deficient in the blocks, only a narrow rim of tissue being left adherent to the anterior lobe.

Anterior pituitary. The anterior pituitary presents a normal architecture, and the relative proportion of cells appears to be within normal limits with differential staining. Rasmussen [1929, 1933] has given methods of differential counting of the cells of the anterior pituitary, and has set normal variations. The method is strictly applicable only to horizontal sections of the gland. Therefore a Rasmussen count cannot be accurately copied in this case. Counting 17,000 cells by the method that Rasmussen defines, but using vertical sections stained by a modified Mallory method, the following results have been obtained: basophil cells, 10%; acidophil cells, 38%; chromophobe cells, 51%. These figures are well within Rasmussen's normal limits, and this was the impression gained on comparison of the sections with sections from 120 other human pituitary glands.

The basophil cells. The basophil cells show three abnormalities: hyalinization, excessive vacuolation, and an increase in size. Only one to two normal granular basophil cells were seen in most of the sections (80) examined.

Basophil-cell hyalinization. As Crooke described, the progressive development of hyalinization, in the cells uncomplicated by excessive vacuolation, is seen to follow an invariable rule when it encroaches upon the granular cytoplasm. Beginning as a

narrow complete, or almost complete, zone situated peripherally, the hyaline change advances towards the nucleus. In most cells showing advanced hyalinization, granular cytoplasm persists in two areas, one in contact with the nucleus on that side of the nucleus which has the most abundant cytoplasm (juxta-nuclear granulation), and another on the periphery of the cell. When eventually the juxta-nuclear granulation is hyalinized the peripheral granulation is often still partially persistent (see McLetchie [1944] for illustrations). All stages from a narrow subperipheral hyaline zone to complete hyalinization are present. About 5 % of the basophil cells are completely hyaline (Plate 1, fig. 1; Plate 2, fig. 6). Cells with a narrow complete subperipheral hyaline zone are particularly numerous; this type does not appear to have been observed by Crooke (Plate 2, fig. 5).

Vacuolation in the basophil cells. About one-fifth of the partially hyaline basophil cells show no vacuolation of the remaining granular cytoplasm. The rest show varying degrees of vacuolation of the granular cytoplasm and in most it is completely vacuolated. Excessive vacuolation in the individual cells is not represented by a large single vacuole but is always of multifocal origin, being represented by a conglomeration of small vacuoles. These may be outlined by a layer of normal ripe granules (intergranular vacuolation) (Plate 2, fig. 4 and fig. 5), but in many the outlining granules have become scanty and are small and feebly stained. Refractile cytoplasmic threads are then revealed outlining the vacuoles, and to this appearance we give the name 'cobweb vacuolation'. This cobweb vacuolation develops in any area of the cell but is most common in the juxta-nuclear area. Cobweb vacuoles often indent and compress the nucleus, which may eventually be flattened out against the periphery of the cell. Depending on the plane of section many configurations are produced (Plate 2, fig. 6 and Plates 3 and 4). Where cobweb vacuolation abuts on hyaline cytoplasm the threads outlining the vacuoles appear continuous with the hyaline cytoplasm. A few very large (35μ) non-hyaline cells are present.

Intrahyaline vacuoles are present in many of the cells with advanced hyalinization (Plate 1, fig. 1 and Plate 3). Cobweb vacuolation is also conspicuous in non-hyalinized cells; stages in its development are shown in Plate 2, fig. 5.

The amount of basophil-cell vacuolation is much greater than in any of the glands of the control series both as regards the proportion of vacuolated cells and the degree of vacuolation in the individual cells.

The nuclei of the basophil cells. In hyaline basophil cells uncomplicated by excessive vacuolation, the nuclei appear normal except for a few of the completely hyalinized cells in which the nucleus is hyperchromatic and slightly distorted. The hyaline cytoplasm of the latter cells stains very deeply. The nucleus may be normal in appearance in cells with excessive cobweb vacuolation, but in the majority it is peripheral and compressed and scalloped by the vacuoles. Despite the remarkable degree to which scalloping of the nucleus may proceed in association with cobweb vacuolation, it is indeed remarkable that we have not seen a single example of nuclear pyknosis. In connexion with the assessment of nuclear pyknosis in the normal anterior pituitary, basophil nuclei vary greatly in chromatin content, some being of dense structure and highly fuchsinophil, and only slight detail is made out with well-differentiated staining and high-power observation. When the latter type of nucleus is compressed and scalloped little nuclear detail is discernible (Plate 3). The nucleoli of some of the nuclei



Fig. 1. Two hyaline basophil cells, the upper completely hyaline with some intra-hyaline vacuoles, the lower with persisting granulations especially in the juxta-nuclear zone.



Fig. 2. A hyaline basophil cell with a single vacuole in the persisting granulation.

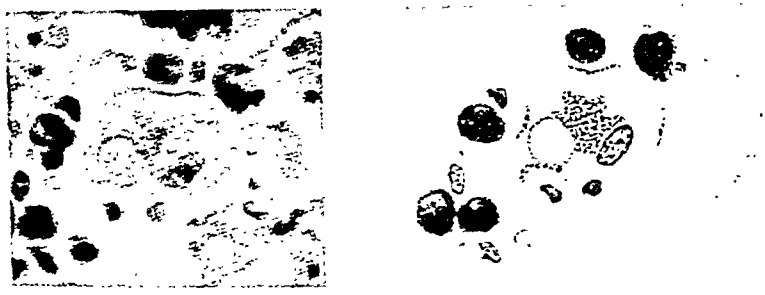


Fig. 3. A large hyaline basophil cell 'cupping' three immature cells; two appear embraced by lateral pseudopodia, the third appears surrounded by the cytoplasm of the parent cell. Note the small size of the surrounding acidophil cells (orange).

All figures × 800, with coloured drawings. Modified Mallory [method IIA; McLetchie, 1944]. Basophil granules, red; hyaline cytoplasm, blue.



FIG. 4. A basophil cell with peripheral hyaline zone and the remainder of the cell taken up by gross intergranular vacuolation.

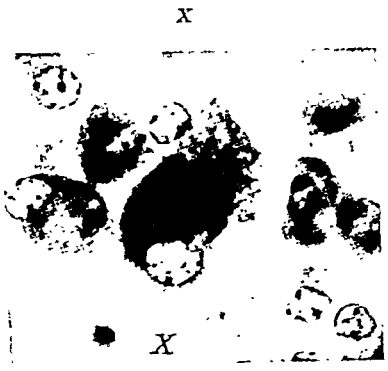


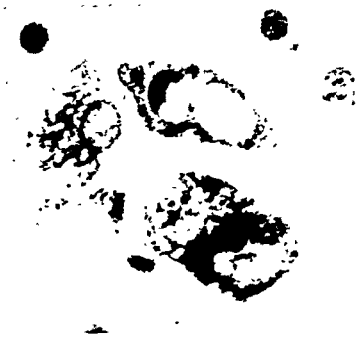
FIG. 5. The development of cobweb vacuolation: *A*, normal basophil cell; *B*, vacuolation developing in 3 main foci, the nucleus is peripheral; *C*, a fully developed cobweb area; *D*, a minute peripheral hyaline zone is present. The two small cells are acidophil cells.

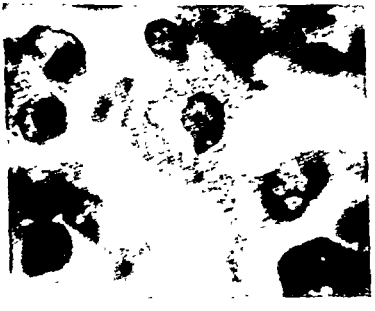


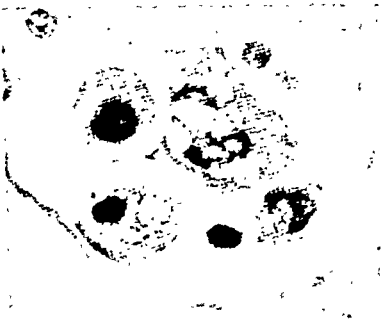
FIG. 6. Scalloping of the nucleus by cobweb vacuoles in a partially hyaline cell; two completely hyaline cells on right.

All figures × 800, with drawings of enlargements. Modified Mallory [method IIA; McLetchie, 1944].



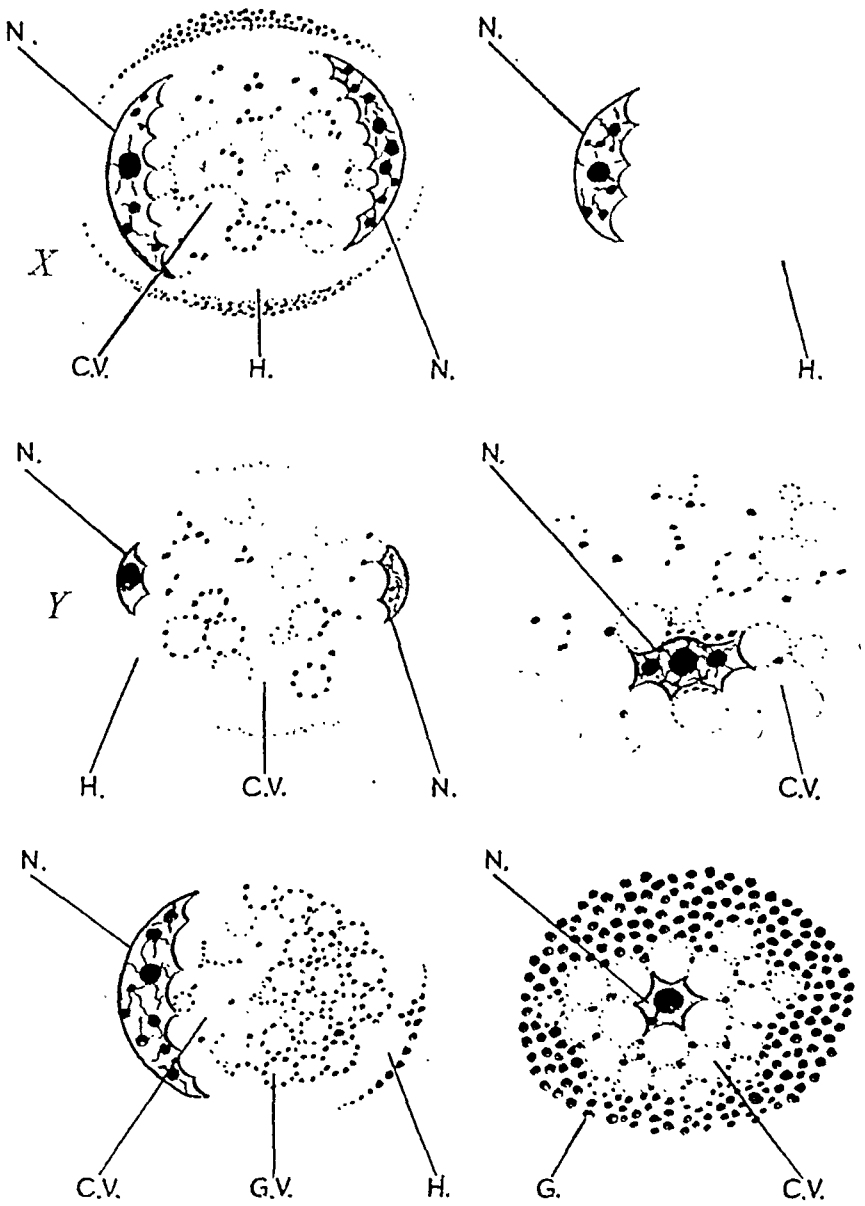






Compare control (*X*) showing normal granular basophil cells with other figures showing abnormal basophil cells with hyalinization, cobweb vacuolation and nuclear scalloping. × 1000. Modified Mallory [method IIB: McLetchie, 1944].

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Diagrammatic illustration of complex basophil-cell abnormalities.

G, normal granularity; *H*, hyaline cytoplasm; *C.V.* cobweb vacuolation; *G.V.* intergranular vacuolation; *N*, nucleus. Cells *X* and *Y* are binucleate. *Y* represents a tangential section of a large cell similar to *X*; note all that is seen of the nuclei in *Y*.

of vacuolated cells appear unusually large. Many binucleate and trinucleate cells are present (4–6 in every section examined). No mitotic figures were found.

Many peculiar dispositions of hyaline, vacuoles, normal granularity and nucleus in the basophil cells give the impression that constant alteration in the morbid process is occurring.

The acidophil cells show normal granularity.

Size of chromophil cells. With regard to the size of the cells, there is a striking abnormality present. In every field examined the diameter of the acidophil cells has been only a half to a third the diameter of the basophil cells; frequently the disproportion has even been greater, while only in a few fields was an acidophil cell found as large as the smallest basophil cell (Plate 1, fig. 3).

In the glands of subjects, aged 20–40, in the control series, acidophil cells vary from 6 to 24μ, basophil cells from 8 to 20μ, in diameter. Acidophil cells show great variation in size; the basophil cells are much more constant, the vast majority being 14–16μ in diameter. Occasionally a giant chromophil cell is seen. In no case has any disproportion in the basophil/acidophil ratio been found approaching the conditions in the present case. Here less than 1% of the acidophil cells are over 12μ; 0·2% have diameters over 16μ; many are less than 8μ. The majority of the basophil cells are 18–22μ in diameter, and about 4% are giant forms of 28–36μ; many of these forms show the phenomenon of cupping (Plate 1, fig. 3; see Severinghaus [1938] for explanation). The table shows the results obtained on measuring, with the eyepiece micrometer, approximately 1000 acidophil, and 1000 basophil, cells from the present case and, as a control, from sections of the hypophysis of a normal adult male, age 34. Both case and control appeared to have the same degree of post-mortem change and shrinkage.

	Diameter of chromophil cells (μ)				
	Over 20	20–16	16–12	12–8	<8
Percentage of basophil cells present in various groups					
Case of Cushing's syndrome	27	30	42	3	0
Control	7	29	52	12	0
Percentage of acidophil cells present in various groups					
Case of Cushing's syndrome	0	0·2	0·7	78	21
Control	3	19	27	47	3

Large series of cells were grouped according to various size ranges corresponding to fixed divisions of the eyepiece micrometer, and measurement was also carried out by projecting the cells on cardboard screens and cutting out and weighing the images. The results were compared with glands from normal adults. Every method of measurement showed: (1) a greater preponderance of basophil cells of larger type than normal; (2) a constant abnormality of the acidophil cells, in that they were all of small type. The chromophobe cells are all of the small type found in the normal adult. No adenoma has been found on serial section.

The posterior lobe. Observation is limited to a rind of tissue attached to the anterior lobe. The wandering cells are plentiful and of normal type. A few show intermediate stages of hyalinization and vacuolation. The pars intermedia is small and presents no abnormality.

Summary of anterior pituitary findings.

(1) A conspicuous degree of basophil-cell hyalinization.

(2) Excessive basophil-cell vacuolation, with development of the cobweb and scalloping of the nucleus.

(3) Enlargement of basophil cells.

(4) Great decrease in size of acidophil cells.

(5) Relative proportion of cells within normal limits.

(6) No adenoma present.

THE CONTROL SERIES

Six to twelve sections from different levels of each gland of a control series of 120 hypophyses were examined by the methods previously described [McLetchie, 1944]. The glands were taken from routine autopsies on adult subjects mainly from medical wards. The sections were specially examined with regard to hyalinization and vacuolation.

Basophil-cell hyalinization. Hyalinization was found to a slight degree in a few (1–3) basophil cells in sections from five different glands. The finding was not constantly associated with any condition. Basophil-cell hyalinization was not found in any of the cases of the series with endocrine abnormality, in many of which the anterior pituitary was abnormal: thyrotoxicosis (3), almost complete ablation of the thyroid by amyloidosis (1), myxoedema (1), islet-cell tumour of the pancreas with hypoglycaemia (1), diabetes mellitus (7), adreno-cortical hyperplasia in a case of hypertension (1), Addison's disease (2), thymic tumour associated with myasthenia gravis (1); two cases of haemochromatosis with marked haemosiderin deposits in the cells of the anterior pituitary. A large number of sections from these cases were examined.

Basophil-cell vacuolation. The observation of cytoplasmic threads outlining basophil-cell vacuoles does not appear to have been previously described. Since it is so conspicuous a feature in the case it might be questioned if this was a special type of vacuolation only associated with the process of hyalinization.

Vacuolation of the basophil cells is present in all the glands of the control series in very varying degree. It is generally more conspicuous in elderly subjects. The vacuolation is mainly of intergranular type, i.e. the vacuoles are outlined by a rim of granules and no enveloping cytoplasmic membrane (refractile thread, in section) is visible, and this obtains even when the vacuolation in individual cells is multifocal. Bearing no relation to the degree of vacuolation in the whole gland or in the individual cells cobweb vacuolation is present in a considerable number of the control series but in very slight degree and in very few cells. It is represented by a few contiguous vacuoles with partial disappearance of surrounding granules and appearance of the cytoplasmic thread. Occasionally, when juxta-nuclear, the cobweb vacuoles scallop the nucleus. The cobweb vacuolation is not associated with hyalinization and its occurrence in the individual cases cannot be attributed to any common factor. The greatest degrees of vacuolation are present in elderly subjects. Severinghaus [1938] has already recorded this and considers that it is a reaction to hypogonadism (senile castration). In none of the elderly subjects in the control series is the basophil vacuolation nearly as marked as it was in the case of basophilism. Inquiry into the contents of the basophil vacuoles has not been made. Biggart [1934], using frozen

sections, found basophil vacuoles to contain an unstainable substance and small quantities of fat.

FURTHER CASES OF BASOPHILISM

In addition to the present case I have found a conspicuous degree of hyalinization of the basophil cells in two other cases of basophilism. One was associated with a large adenoma of ripe basophil cells (5 × 6 mm.). The cells of the adenoma showed normal granularity without hyalinization; one-third of the basophil cells of the surviving gland showed varying degrees of hyalinization, but mainly minute subperipheral rims. The other case was associated with an adreno-cortical carcinoma. Basophil-cell hyalinization was very marked. There was no abnormality in the size of the chromophil cells in either case, the degree of basophil-cell vacuolation was well within normal limits and there was no development of cobweb vacuolation. The basophil-cell nuclei appeared normal and no nuclear scalloping was found.

DISCUSSION

I intend to attempt first to evaluate the various hypophyseal abnormalities found in the case as far as possible in the light of what is known of pathological reactions in the human hypophysis, and secondly to discuss the findings in relation to allied experimental work.

The pathological findings in this case of Cushing's syndrome are unusual in that there is neither hypophyseal, adreno-cortical nor thymic tumour, nor adrenal hyperplasia. Such a pathological type of basophilism is rare but not unknown [Oppenheimer *et al.* 1935; Freyberg *et al.* 1936; Ulrich, 1936]. Nevertheless, the hypophysis is highly abnormal. In addition to a conspicuous degree of basophil-cell hyalinization, the hypophysis shows: basophil-cell enlargement; excessive basophil-cell vacuolation, associated with degranulation and the appearance of cytoplasmic envelopes round the vacuoles, which displace, compress and scallop the nucleus; and marked decrease in the size of the acidophil cells.

There is no true indication in the literature of basophilism as to the incidence of the latter abnormalities, owing to the cursory examination and scanty description of the anterior pituitary in many cases.

The excessive basophil-cell vacuolation

Basophil-cell vacuolation is associated with disappearance of β granules, and I consider that the appearances in this case and the control series indicate that cobweb vacuolation is merely an intensification of the usual process where, with increased disappearance of surrounding granules, there is revealed a refractile cytoplasmic envelope (thread in section) surrounding the vacuoles. I consider that it is probable that in 'intergranular' vacuolation the envelope is also present but is masked by the surrounding granules.

It appears unlikely that the excessive disappearance of β granules associated with the excessive vacuolation and cobweb development can be attributed to the hyaline cytoplasm in the cells complicating the normal process of vacuolation, in that the process is also extreme in non-hyaline basophil cells in the same gland and is present in the control series unassociated with hyalinization or with any other obvious abnormal factor.

Though I am convinced that cobweb vacuolation is merely a further development of ordinary intergranular vacuolation and is therefore cytoplasmic and not nuclear, Severinghaus [1938], Rasmussen [1936] and Graef, Bunim & Rottino [1936] have interpreted what appear to me to be similar appearances in hyaline basophil cells in Cushing's syndrome as nuclear 'blisters'.

Severinghaus [1938], commenting on Crooke's findings, states (p. 84) that on examining sections from a case (Graef's) of Cushing's syndrome in 1934, 'I reported similar changes, which I characterized as a general degranulation of the basophils of the hypophysis....Nuclear changes in these basophils were to me, however, more striking than the cytoplasmic. Very few cells had nuclei. In most cases they were extremely lobulated, frequently with vesicular bulges, not unlike thin-walled blisters which occupied most of the volume of the cell. Deeply shrunken, elongated, pyknotic nuclei were numerous.' Therefore Severinghaus clearly interpreted the vacuoles as nuclear structures. In the diagrammatic illustrations provided the artist has been tardy to follow the written descriptions and at the most has drawn the vacuoles as herniations of the nucleus, the nuclear membrane being drawn mainly intact.

Rasmussen reported that in the anterior pituitaries of three cases of Cushing's syndrome, in addition to basophil-cell hyalinization, 'the nuclei of these abnormal cells may be enlarged and lobulated as if ballooned out in several places. These nuclear changes have been stressed by Severinghaus....The nuclei of some of these cells may appear essentially normal.'

Graef *et al.* state that the nuclear blisters, 'exhibited no chromatism with any of the stains employed'.

Cobweb vacuolation can be easily made to simulate nuclear 'blistering'. With the staining methods used [McLetchie, 1944] nuclear and cytoplasmic structures have always been brilliantly differentiated. The process of cobweb vacuoles compressing the nucleus is obvious when the plane of section passes through the long axis of the crescentic scalloped nucleus, but when the plane is tangential only a small area of indented nucleus is present. In many staining methods advocated for the anterior pituitary, the nucleus, granules and hyaline cytoplasm of the basophil cells appear as slightly different shades of blue. The lightly stained cytoplasmic threads of the vacuoles appear, with these stains, as continuations of the nuclear chromatin threads. Cobweb vacuolation is then easily mistaken for a continuation of nuclear structure.

I have always found that persisting granules studding the threads which outline the vesicles give the reactions of cytoplasmic and not nuclear material, and I am of the opinion that the appearances are completely explained as a result of compression and scalloping of the nucleus by the cobweb vacuoles.

Diminution in size of the acidophil cells in basophilism has only been recorded once before, as far as I can ascertain—in the Rabbe-Krause case of Cushing's original series [Cushing, 1932]. The decrease in size was not so marked as in the present case.

The significance of the abnormalities of size and vacuolation of the chromophil cells

It cannot be held that these abnormalities of size and vacuolation present in the chromophil cells of the hypophysis represent a congenital anomaly of the hypophysis, in that the subject was a normal healthy virile male until three years before death and was of normal stature. Furthermore, these abnormalities must have only some

secondary significance in relation to the morbid process of basophilism, in that they are inconstant findings in basophilism (I did not find them in the two other cases of basophilism mentioned); and they are not associated with any particular pathological type, since similar vacuolation was found in Graef's case of adreno-cortical carcinoma, and decrease in size of acidophil cells occurred in the Rabbe-Krause case of basophil adenoma.

Castration during active sexual life is the only condition which is known to produce in the human hypophysis a degree of basophil-cell vacuolation similar to that found in the present case [Biggart, 1934]. In the castrated rat, in addition to excessive basophil-cell vacuolation, the basophil cells increase in number and size, while the acidophil cells decrease in number and size [Severinghaus, 1938]. In the hypophysis of the castrated human, of which few critical examinations have been made, only excessive basophil-cell vacuolation and perhaps basophil-cell increase have been reported [Biggart, 1934]. There is no detailed account of the type of vacuolation, though Rasmussen [1938] depicts it as a single large vacuole taking up the bulk of the cell. I have reason to believe that this is by no means always the case, in that in the virtually castrate state of senility, as seen in the aged subjects of the control series, any excessive basophil-cell vacuolation is not represented in this form in the large number which we have examined, but is represented by a conglomeration of small vacuoles in the basophil cells specially affected.

The castrate state and Cushing's syndrome have much in common clinically, while gonadal atrophy and fibrosis are frequently present in Cushing's syndrome [Cushing, 1932]. Unfortunately, no microscopic examination of the testes of my case was made.

I consider that the increase in size and excessive vacuolation of the basophil cells and the decrease in size of the acidophil cells are likely to be correlated with the findings in the castrated rat and are to be interpreted as representing a phase of hypophyseal reaction secondary to hypogonadism—and I think that this explanation probably also applies to similar changes produced in the *Brassica* response, which is referred to later, and of which hypogonadism is a feature. Why these changes should be inconstant in basophilism is unknown, but not incompatible with our knowledge of the anterior pituitary, in which change, even in morbid processes, is probably constantly occurring. An example of this is the finding by Severinghaus of a high basophil-cell count in the hypophysis from a case of Addison's disease as opposed to the constant finding by others of great basophil-cell decrease in this condition. The unusual finding is interpreted as a consequence of death occurring during a phase of basophil-cell regeneration, which must be rare in this condition [Severinghaus, 1938].

There is possibly another factor concerned with the increase of size of the basophil cells; while many of the very large ones show gross vacuolation, others, unassociated with vacuolation, show an advanced degree of hyalinization. The latter cells have frequently two or more nuclei, and we consider that their enlargement is probably more correctly to be interpreted as a manifestation of functional hypertrophy as distinct from the enlargement associated with gross vacuolation.

The hyalinization of the basophil cells

While the abnormalities of chromophil-cell size and vacuolation must be considered as secondary changes bearing a very general interpretation to the morbid process of

Cushing's syndrome, this cannot be said of the conspicuous degree of basophil-cell hyalinization which is present.

Crooke considers that hyalinization of the basophil cells is an indication of altered physiological activity, as opposed to a stage of cell death, since the basophil-cell nuclei are entirely normal. Severinghaus [1938] contends that it is a stage to cell death since he found nuclear breakdown (blistering) associated with hyalinization. My interpretation of the nuclear appearances favour Crooke's view as to the essential nature of the lesion. Moreover, it is apparent from the examination of the other two cases of basophilism that basophil-cell abnormality additional to hyalinization is inconstant.

The presence of basophil-cell hyalinization has never been denied in any case of Cushing's syndrome reported since Crooke's original communication, except in one instance—a case described by Pons & Pappenheimer [1937]. I do not consider that it is clinically a case of basophilism and, moreover, only one-half of the gland was examined and basophil cells were scanty in that half. Basophil-cell hyalinization has been found in two cases of basophilism associated with unique pituitary lesions, not included in Crooke's original series—a metastasizing basophil carcinoma [Cohen & Dible, 1937], a large chromophobe adenoma [Fuller & Russell, 1936]. I now add this third type—a conspicuous degree of hyalinization of the basophil cells in a case of basophilism without tumour. Rasmussen [1938] has already made very brief reference to basophil-cell hyalinization in such a type.

These findings contrast with the inconstant and exceptional finding of a few hyaline basophil cells in conditions other than Cushing's syndrome [Crooke, 1935; Rasmussen, 1936; my control series], and especially with the absence of basophil-cell hyalinization in all cases of endocrine abnormality in the control cases. All these substantiate Crooke's hypothesis that the abnormal activity of the basophil cells, of which hyalinization is the morbid manifestation, is the essential abnormality of Cushing's syndrome. Special weight is added to this view by the present case in which no tumour or hyperplasia of the basophil cells of the hypophysis, the adrenal cortex or of the thymus is present, and in which the peculiar nuclear appearances in many basophil cells are accounted for by the effects of vacuolation in the cells. Certain other considerations to be discussed later may modify this view of the significance of hyalinization.

The interrelationship of the different pathological types of Cushing's syndrome

Many writers have taken definite views either that basophilism is essentially a hyper-adreno-cortical or a hyper-basophil condition [see Pardee, 1938]. While neither view is justified on morbid anatomical grounds when all the pathological types are considered, attention must be drawn to the correlation existing between many of the pathological types of basophilism in addition to the common bond of basophil-cell hyalinization. Thus, it is the rule rather than the exception to find adreno-cortical hyperplasia associated with basophil adenoma [Freyberg *et al.* 1936]; and again in some cases of adreno-cortical tumour, basophilia, that is a relative increase in the basophil cells, is present [Lescher & Robb-Smith, 1935; McLetchie & Scott, 1944]. In McLetchie & Scott's case basophil cells are actively developing from the 'ducts' of the pars intermedia in contrast to the other cases of basophilism which I have

examined and the glands of the control series, in none of which is such active basophil-cell increase evident.

The above findings suggest that hypersecretory processes of the basophil cells and the adrenal cortex are complementary, the one producing the other.* Since an organ need not necessarily enlarge with hyperactivity, I consider that in those cases of basophil adenoma and adreno-cortical tumour in which there is not enlargement of the complementary organ (adreno-cortical hyperplasia, basophilia) either hyperactivity of the complementary organ is present but is not expressed in enlargement or the organ was not in a phase of hyperactivity at the time of death, but was so earlier.

In Addison's disease, destruction of the adrenal cortex is accompanied by great decrease in the number of basophil cells of the hypophysis [Crooke & Russel, 1935]. These facts suggest that the basophil cells and the adrenal cortex constitute a functional complex. Cushing's syndrome is in almost every respect an opposite condition to Addison's disease [McQuarrie, Johnson & Ziegler, 1937]. One might therefore claim that while Addison's disease is the symptom of hypofunction of the basophil-adreno-cortical complex, Cushing's syndrome is a symptom of hyperactivity of the complex.

The thymic tumours of Cushing's syndrome are associated with adreno-cortical hyperplasia. Thus whether in Cushing's syndrome there is a basophil adenoma, adrenal tumour or thymic tumour, there exists sufficient correlation on morbid anatomical grounds to indicate the eventual production of a common inter-endocrine abnormality quite apart from the finding of basophil-cell hyalinization. But in the case which I have described there is no absolute morbid anatomical evidence of basophil, adreno-cortical or thymic hyperactivity. However, it must be kept in mind that the pathological findings in the hypophysis of basophilism are only those obtaining at a point of time in a complex morbid process possibly undergoing constant alteration. I have already noted that, in the present case, although the basophil cells were not increased in number, large binucleate and trinucleate forms were numerous. A few binucleate (and trinucleate) basophil cells are encountered in most human hypophyses. The proportion is very variable throughout any gland and no definite statement could be given about the proportion in the present case, with reference to normal, without a very extensive statistical survey. Nevertheless, inspection of the sections gives the impression that large binucleate forms are abnormally numerous. It is possible that this is an indication of basophil-cell hyperactivity.

Thus apart from the common bond of basophil-cell hyalinization the rather unique case of basophilism described offers possible correlation with the common morbid anatomical types of basophilism in that there is evidence suggestive of basophil-cell hyperactivity. The final elucidation of the problem depends on the significance of basophil-cell hyalinization. While this problem must ultimately rest with the experimental worker, I consider that certain observations at least suggest a solution, though the conclusions reached must be taken with reserve as the evidence is based on histological change from a quantitative aspect which is never a certain guide.

* The relation of adreno-cortical tumour to the adreno-genital syndrome and to Cushing's syndrome is dealt with elsewhere [McLetchie & Scott, 1944].

The significance of basophil-cell hyalinization

Crooke has noted two special points with reference to the basophil adenoma of Cushing's syndrome: (1) the cells of the adenoma did not show the hyaline change; and (2) while the basophil cells of the surviving parenchyma showed hyalinization, it was not so conspicuous as in the hypophyses from other morbid types of basophilism (adrenal tumour, thymic tumour) in five of the six cases of basophil adenoma examined by him. This was also the finding in the case, previously referred to, where no hyalinization was present in the large adenoma while hyalinization was mainly limited to minute subperipheral rims in about one-third of the basophil cells of the surviving parenchyma. In the case reported by Cohen & Dible [1936] a metastasizing basophil carcinoma was associated with a clinical syndrome containing practically every sign ever recorded in Cushing's syndrome. Hyalinization was not present in the cells of the carcinoma. In the very scanty surviving gland parenchyma hyalinization was present in slight degree in a very few basophil cells and was demonstrated with difficulty [Cohen & Dible, 1937]. In the first place it would appear that the cells of the basophil adenoma of Cushing's syndrome do not obey the same laws as the basophil cells of the parenchyma in that they do not undergo hyalinization—a factor perhaps not inconsistent with our knowledge of the autonomy of tumour cells. Secondly, especially from the evidence of Cohen & Dible's case, it would appear that the basophil tumour, when present, is the significant abnormality in the hypophysis and that basophil-cell hyalinization is a subsidiary phenomenon since the latter may be relatively inconspicuous. Since this evidence indicates that hyperactivity of the basophil cells is the essential lesion in basophilism, and since basophil-cell hyalinization is a constant finding in all pathological types of basophilism, it would be reasonable to assume that cytoplasmic hyalinization is a change consequent on basophil-cell hyperactivity. As we shall see later this assumption goes far to unify all the varied pathological pictures which pituitary basophilism is heir to, and is consistent with the experimental production of basophil-cell hyalinization in association with antihormone production [Severinghaus & Thomson, 1939].

Before attempting to evaluate the significance of the experimental production of hyalinization of the basophil cells in the *Brassica* response in the rat it is advisable to summarize my explanations of the various findings in Cushing's syndrome.

Mechanism of pituitary basophilism

Basophilism may be initiated by a hypersecretory tumour of the basophil cells of the hypophysis, of the adrenal cortex, or of the thymus. The basophil adenoma produces adreno-cortical hyperfunction, which is usually expressed in enlargement of the adrenal cortex. The adreno-cortical tumour produces basophil-cell hyperactivity which may be associated with active increase in basophil cells (basophilia). The interglandular relationships of the thymus are obscure, but the mechanism in Cushing's syndrome is understandable in that adreno-cortical hyperplasia and basophil-cell hyalinization are constantly associated with the thymic tumour of Cushing's syndrome. Basophil-cell hyperactivity, which is common to all these mechanisms, acting on all other endocrine glands results in the multiglandular disturbance, the outward manifestation of which is Cushing's syndrome. Basophil-cell hyalinization is a cytoplasmic

change consequent on hyperactivity of the basophil cells and hence is common to all these mechanisms. In the case of the basophil adenoma the tumour cells do not obey the ordinary laws applicable to the basophil cells of the hypophysis and do not develop hyalinization. The reaction of the basophil cells of the surviving gland in the hypophysis containing a basophil adenoma is only of secondary significance and only slight hyalinization is usually produced. In some cases, such as the one we have described, no tumour is present. It would appear reasonable to assume that in these cases there is a generalized basophil-cell abnormality in which phases of hyperactivity alternate with hyalinization. Consequent on the multiglandular abnormality produced by basophil-cell hyperactivity certain secondary changes may occur in the hypophysis: excessive basophil-cell vacuolation, decrease in size of acidophil cells, focal chromophobe-cell hyperplasia [see McLetchie & Scott, 1944, for the latter]. The excessive vacuolation of the basophil cells with cobweb development and consequent nuclear distortion may be, at some phases of the disease, one of the most conspicuous morbid appearances in the hypophysis.

The Brassica *response in the rat; its relation to the morbid process of pituitary basophilism*

Goitrogenic factors have been shown to be present in a number of vegetable sources, principally in the *Brassicae* (rape and turnip seeds) [Chesney, Clawson & Webster, 1928; Hercus & Purves, 1936; Sharpless, 1938]. Kennedy, Griesback & Purves have shown that the following changes are produced in rats by feeding *Brassica* seeds.

(1) An initial rapid proliferation of the thyroid in the first two weeks; after the third week the thyroid growth parallels the growth of the rat [Kennedy & Purves, 1941].

(2) The basophil cells of the hypophysis increase rapidly in size and number (within 7 days), cytoplasmic hyalinization develops (14 days) and later is complicated by excessive cytoplasmic vacuolation (28 days). By the 56th day, hyalinization and vacuolation are maximal, with the production of many signet-ring cells. All these changes regress and by 224 days the basophil cells approach normality with little vacuolation or hyalinization, though some basophilia persists.

(3) The acidophil cells of the hypophysis decrease in number and size for a period, but by 212 days the acidophil-cell picture is normal [Griesbach, 1941].

(4) The adrenal cortex enlarges and the lipoid content is increased; the development of the ovaries is retarded; growth continues but at a suboptimal level [Kennedy & Purves, 1941].

Hypophysectomy can either prevent a *Brassica* response or cause a previously induced response to regress rapidly [Griesbach, Kennedy & Purves, 1941].

The authors appear to be mainly concerned with the correlation of the hypophyseal and thyroid changes and give little detail of other aspects. Their illustrations are convincing and the basophil 'hyaline' to which they refer appears to be similar to that of Crooke; unfortunately, they do not discuss this important point.

The *Brassica* response in the hypophysis at 56 days corresponds to our findings in the present case of basophilism except that basophilia is not present in the latter. Furthermore, the initial *Brassica* responses, basophilia and adreno-cortical hypertrophy, correspond to what appear to be common precursors of basophil-cell hyalinization in the human subject. Thus I consider that the findings in the response have much wider implications than as a function of thyroid activity.

While the morbid changes in the *Brassica* response take place over a relatively large period of a rat's life they ultimately regress and the hypophysis appears to undergo a single cycle of change. Multiglandular upset is produced but not of such a degree as in Cushing's syndrome, though it is similar, thyroid enlargement, adreno-cortical hyperplasia and gonadal atrophy being common findings in basophilism. While basophil-cell hyalinization in the *Brassica* response is rapidly produced and long continued, the multiglandular changes appear to occur in the short initial stage of basophilia before hyalinization is marked. This is certainly true of the thyroid alteration; unfortunately the authors do not give the time relations of the adreno-cortical changes. Thus it would appear that the essence of the *Brassica* response is a short phase of basophil-cell hyperactivity productive of multiglandular disturbance while the prolonged phase of hyalinization is the morbid manifestation of recovery from basophil-cell hyperactivity and in itself does not contribute to endocrine derangement, i.e. it is a purely reparative phenomenon. In Cushing's syndrome the phase of hyperactivity of the basophil-cell adreno-cortical complex is often continuous in the form of a hypersecretory tumour of the basophil cells or of the adrenal cortex, while in the cases without tumour it is probable that we have phases of increased basophil-cell activity alternating with hyalinization; at any rate there is no evidence that the condition regresses completely. I do not consider that the morbid process of the *Brassica* response and of pituitary basophilism can be separated qualitatively, and it may well be that with further research the initial effects of the *Brassica* response may be so heightened or frequently repeated that a more exact correlation with the human disease may be produced. If it is correct to correlate this experimental work with the hypophyseal findings in the present case it would indicate that:

(1) the hypophyseal changes found in the present case are a consequence of hyperactivity of the basophil cells;

(2) it is the phase of basophil-cell hyperactivity which is the essential factor in the production of endocrine upset;

(3) basophil-cell hyalinization is a constant finding in Cushing's syndrome because it is a cytoplasmic change resulting from hyperactivity of the basophil cells.

SUMMARY

1. A case of basophilism, unassociated with hypophyseal, adrenal or thymic tumour and without adrenal hyperplasia, is described.

2. In the anterior pituitary the following abnormalities are present:

(*a*) a conspicuous degree of basophil-cell hyalinization;

(*b*) an excessive degree of basophil-cell vacuolation;

(*c*) multifocal basophil-cell vacuolation in the individual cells is associated with excessive disappearance of granules and with the revelation of a refractile cytoplasmic envelope surrounding the vacuoles (cobweb vacuolation);

(*d*) the nuclei of the basophil cells are normal but in many cells cobweb vacuolation is associated with displacement of the nuclei to the periphery of the cells and with compression and scalloping of the nucleus by the vacuoles; binucleate basophil cells are numerous;

(*e*) the basophil cells are increased in size;

(*f*) the acidophil cells are greatly reduced in size.

3. These findings are contrasted with the findings in (*a*) two other cases of basophilism, (*b*) a large series of hypophyses from non-basophilism cases and (*c*) other recorded descriptions of the abnormal basophil cells of basophilism.

4. On the basis of the evidence gathered, it is pointed out that:

(*a*) the abnormalities of size and vacuolation found in the chromophil cells are: (i) inconstant in basophilism, (ii) not associated with any particular pathological type of basophilism;

(*b*) the cobweb vacuolation and nuclear scalloping in the basophil cells is similar to what Severinghaus and others have interpreted as nuclear 'blistering'.

5. It is suggested that the abnormalities of size and vacuolation in the chromophil cells represent a phase of reaction to hypogonadism, and are only of secondary importance in the morbid process of basophilism.

6. The correlation between the different pathological types of basophilism is briefly discussed.

7. The relation of the morbid process of Cushing's syndrome to the changes produced in rats fed on a diet containing a high proportion of *Brassica* seeds is discussed.

8. It is considered that basophil-cell hyperactivity is the essential abnormality of Cushing's syndrome, and that basophil-cell hyalinization is a cytoplasmic change resulting from hyperactivity.

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Note added in proof. The experiments of Kennedy, Griesbach & Purves referred to in the text have been repeated. Similar results to theirs were obtained in all respects except that neither increase of mature basophil cells nor cytoplasmic hyalinization of the basophil cells in the hypophysis was found.

CARCINOMA OF AN ADRENO-CORTICAL REST ASSOCIATED WITH HYPOPHYSEAL ABNORMALITY

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We record this case not so much because of the rarity of tumours of an adreno-cortical rest but because of the extreme changes present in the hypophysis. While the association of adreno-cortical tumour with hypophyseal abnormality is well known, in no case has such profound hypophyseal change been associated with such a paucity of symptoms. Moreover, we consider the findings, up to a point, capable of explanation.

DESCRIPTION OF CASE

Clinical history

A female shop assistant aged 26 years was seen in July 1940, complaining of an acneform eruption of the face, hirsuties, and coarsening of the features. She had first noticed the acne and the alteration in facial appearance six weeks before. Her previous health had been good and she had no complaints other than concern over the sudden change in her appearance. Her previous menstrual history was normal. She had missed two menstrual periods since the development of the acne. There was no relevant family history.

On examination, the facial features were coarse, the complexion was slightly dusky, and acne vulgaris was present over the face and brow; there was an obvious moustache and a slight growth of hair over the beard area. The hair of the head was normal. A normal degree and distribution of adiposity was present over both trunk and limbs; striae were not seen. The breasts were small. The pubic hair was of female distribution. The external genitalia were normal and there was no enlargement of the clitoris. The blood pressure was 150/90 mm. Hg, and there was no apparent cardiac enlargement. Examination of the abdomen did not reveal any palpable tumour masses.

While awaiting admission to hospital, she reported regularly and the condition appeared to be stationary, but in the sixth week after the original consultation she suddenly became comatose and was sent to the Western Infirmary. On admission she was found to be comatose and could be roused only with difficulty; she refused to speak and resented examination. By clinical examination the liver was found to be palpable one finger-breadth below the costal margin, but no other abnormality was detected. The blood urea was 40 mg./100 ml., and the blood sugar was 80 mg./100 ml. The cerebrospinal fluid was not under increased pressure and contained no increase of protein or cells. The fundi were normal. She remained in this condition for two days, but on the third day the temperature and pulse rate began to rise, a temperature of 103° F. and a pulse rate of 120 beats/min. being reached on the fourth day. On the fifth day she had occasional twitchings of the face and left arm, and died after a generalized convulsion. Post-mortem examination was carried out 12 hr. later.

Post-mortem examination

Apart from the findings in the endocrine glands, which will be described in detail, the following changes were noted. The liver (1500 g.) was permeated with secondary carcinomatous deposits to such an extent that there was little liver tissue remaining in the left lobe. Much of the hepatic tumour was necrotic. A number of lymphatic glands in the neighbourhood of the pancreas were also invaded by secondary tumour growth. The uterus was normal, as were the kidneys apart from slight congestion. The heart (320 g.) showed slight hypertrophy of the left ventricle. The vertebrae and left femur appeared normal on section, fatty marrow being present in the latter and no bony change being evident. A localized area of congestion and dilatation of the superficial veins was present over the right occipital lobe of the brain.

The endocrine glands

On opening the abdomen, considerable fibrous adhesion was noted in the neighbourhood of the pancreas, towards the tail of which a hard nodule could be felt. The capsule of the left adrenal was adherent to the pancreas, and owing to the difficulty of making out precise relations, the pancreas, kidneys, adrenals and aorta were removed together, fixed in Kaiserling's fixative solution and subsequently dissected. The hard nodule was then seen to be a mass of tumour tissue, 2·5 cm. in diameter, replacing the substance of the pancreas near its tail. An irregular fibrous capsule appeared to invest the tumour, separating it from the pancreas medially and from the left adrenal laterally. In order to determine the exact relationship of the adrenal gland to the tumour, large blocks of tissue comprising pancreas, tumour, and left adrenal in continuity were prepared for histological examination. When this was done it was evident that the tumour lay completely outside the capsule of the left adrenal (Plate 1, fig. 1).

The pancreas

The pancreas, apart from the tumour, appeared to be normal. Histological examination of the tumour showed alveoli filled with cubical cells and supported by a delicate fibrous stroma; in some areas the structure resembled that of the zona glomerulosa of the adrenal cortex; in others the cubical cells were arranged in long columns, the structure resembling the zona fasciculata. Thin-walled vessels were present, but the growth was not very vascular (Plate 1, fig. 3). The epithelial cells had finely granular cytoplasm and single, oval, hyperchromatic nuclei; a few large nuclear forms were present but aberrant forms and mitotic figures were not found. The secondary deposits in liver and lymph glands had also this simple adenomatous structure. A dense fibrous capsule invested the primary tumour, but in places tumour cells had infiltrated beyond the capsule and were arranged in masses of small polygonal cells with dense hyperchromatic nuclei and very scanty cytoplasm. In general, the tumour corresponded to the malignant adreno-cortical adenoma described by Ewing [1928].

The adrenals

The adrenal glands were larger than normal, and had notably rounded edges. The right adrenal weighed 12·8 g. and the left 16 g. (allowance being made for a wedge left adhering to the pancreas). The increase in size involved the cortex of each gland



FIG. 1. The primary tumour (pale mass) embedded in pancreas (dark mass at top) and coming into relation with the left adrenal (dark mass at bottom). × 2. Picro-Mallory.

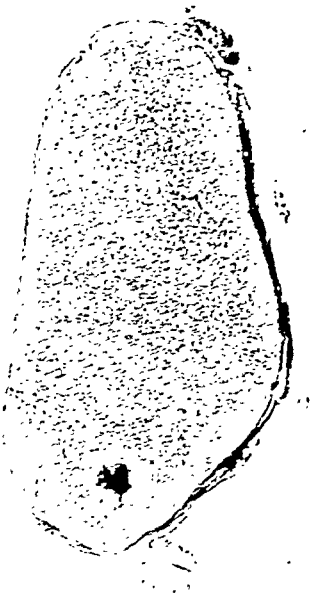


FIG. 2. Adenoma with central mass of colloid in cross section of whole anterior pituitary. × 6. Mallory.

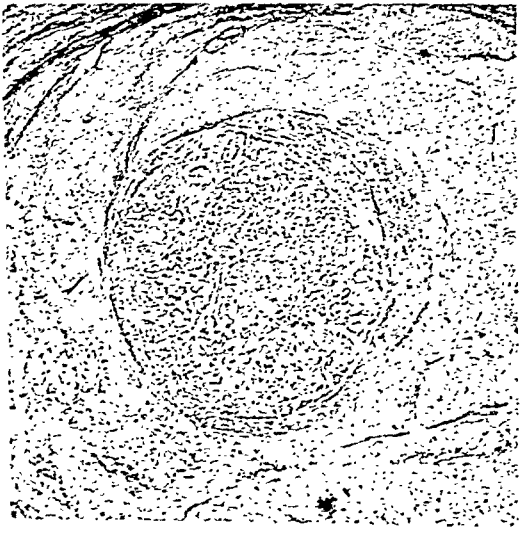
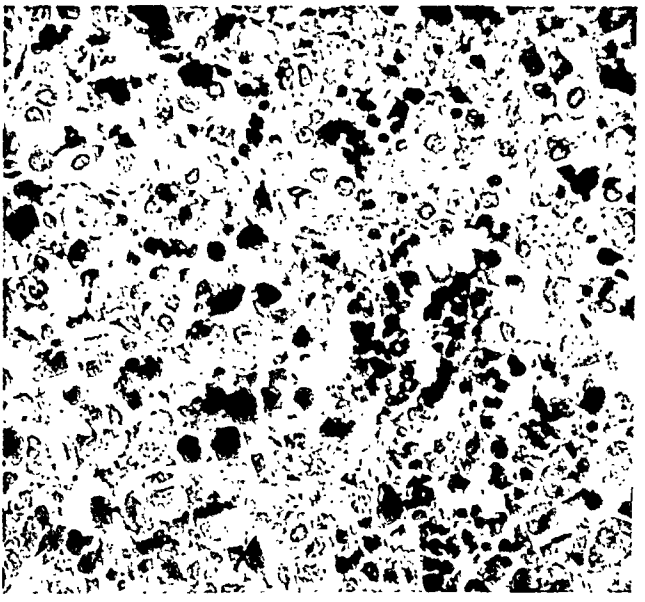


FIG. 3. The primary tumour; note the resemblance to adrenal cortex. × 35. van Gieson.





Control strip.

FIG. 4. Advanced basophil-cell hyalinization in the anterior pituitary. All the pale cells are hyaline basophil cells; note the clarity with which nuclei stand out in hyaline cytoplasm. The dark cells are acidophil cells. Compare with control strip from section of normal gland containing acidophil and basophil cells. × 300. Modified Mallory [method IIA; McLetchie, 1944*a*].

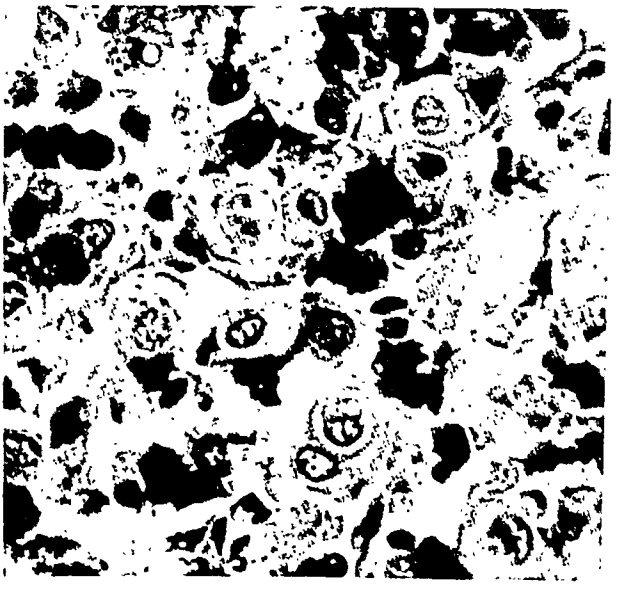


FIG. 5. Enlargement made from an area of Fig. 4. Note few persisting granules (dark areas) at periphery and next nucleus of hyaline cells.

(cortical hyperplasia), and the medulla on each side was normal in size and appearance. The deposition of fat and doubly refracting material in each cortex appeared normal.

Since it has been claimed that the essential evidence of androgenic overactivity in the adrenal cortex is the demonstration of fuchsinophil material in the epithelial cells [Broster & Vines, 1938], a search for this was made in the adreno-cortical tissue, the primary tumour in the pancreas, and the glandular and hepatic metastases. Small amounts of highly fuchsinophil material were found in the epithelial cells in all sites with the exception of the hepatic metastases. In the adrenal cortex the material was present in small scattered areas in all three zones. In the primary tumour and glandular metastases it was found in scattered areas, in some of which it was plentiful, and presented a striking appearance, being disposed in discrete granules and also sharp-edged crescentic inclusions. The fuchsinophil material had no obvious relation to autolytic or necrotic processes, being on the contrary absent in the necrotic and autolysed hepatic secondaries. According to Vines, the substance responsible for this staining reaction is labile and tends to diminish as the result of post-mortem autolysis; it is therefore probable that the fuchsinophil reaction would have been more plentiful had the tissue been fresher, and had the blocks of tissue examined received primary fixation. Apart from general enlargement and the presence of the granules of Vines, we could find no difference in the structure of the adrenal cortex from that of the normal adult cortex.

The pituitary gland

Serial sections of the gland were examined. The histological technique and methods of enumeration and measurement of cells employed are described elsewhere [McLetchie, 1944 *a*, *b*].

The anterior pituitary was of normal size and gross structure.

The basophil cells. The relative number of the basophil cells (14·9 %) was slightly above the maximum normal (13·6 %) and well above the mean for the age and sex of the subject, as defined by Rasmussen [1929, 1933]. Apart from the actual enumeration, the basophil cells appeared much more numerous than in any other hypophyses from young subjects which we have examined. All the basophil cells seemed of large size, but measurement showed that they were in fact within normal limits. The majority of the basophil cells showed the more extreme forms of cytoplasmic hyalinization described by Crooke [1935], the normal granular cytoplasm being replaced by wax-like, homogeneous, refractile cytoplasm. Many of the cells were completely hyalinized, others showed partial hyalinization with granules persisting as a peripheral rim and as small juxta-nuclear collections. The persisting granules were mainly very small and feebly staining. Normal granular basophil cells were rare, only one or two being encountered in an occasional section. The nuclei of the basophil cells appeared normal even in the completely hyaline cells. Basophil-cell vacuolation was inconspicuous. The extreme degree of the cytoplasmic alteration in the basophil cells is evident from the illustrations (Plate 2, figs. 4, 5).

The size and relative proportion of the acidophil and chromophobe cells were well within normal limits. These cells showed no cytological abnormality.

Adenomata

In the inferior part of the gland there were three small adenomata, all about 1·5 mm. in diameter. The term 'adenoma' is used in the conventional sense adopted in hypophyseal histology—a focal accumulation of a single cell type with loss of the normal acinous plan. Two of the adenomata were of papillary structure, the cell type being similar to that of the normal chromophobe cells of the gland. They had indefinite edges merging imperceptibly into normal parenchyma. In the third adenoma there was a central zone of colloid material, similar to the colloid of the cysts of the pars intermedia, surrounded by a cellular zone sharply demarcated from surrounding anterior pituitary structure, with compression of adjacent acini (Plate 1, fig. 2). The cells of the adenoma were loosely arranged, with little stromal support, and with no suggestion of polarity. The cells had large oval vesicular nuclei and scanty irregular, finely granular, cytoplasm; some cells appeared as almost bare nuclei. The staining reaction of the cells differed slightly from the chromophobe cells of the gland and appeared to conform to the 'indeterminate' adenoma described by Graef, Bunim & Rottino [1936] in a similar type of case, but the degree of post-mortem change present does not justify a special description of the tinctorial affinities of the cells. We will refer to the two chromophobe adenomata and the 'indeterminate' adenoma collectively as the chromophobe adenomata.

The pars intermedia was relatively large with many duct-like spaces lined by one or two layers of columnar cells with faintly granular cytoplasm. In places these cells showed differentiation to cells indistinguishable from anterior-lobe basophil cells and many of the ducts were packed with basophil cells, many of which showed hyalinization.

The posterior lobe. The gross structure appeared normal. Wandering basophil cells were numerous in the area adjacent to the pars intermedia. The majority were hyalinized. Deep in the posterior lobe there were two irregular and incomplete gland-like spaces lined by columnar cells similar to those in the pars intermedia. These cells could be seen differentiating into cells indistinguishable from anterior-lobe basophil cells. Wandering basophil cells produced from this area were scattered in the nervous matter around the spaces. Many of these basophil cells showed hyalinization.

Other endocrine glands

The thyroid gland showed normal involutional tissue. In the right ovary primordial follicles, corpora lutea and corpora albicantes were present; the left ovary was represented by a single cyst lined by flattened cubical cells. No thymic tissue was found in the mediastinum.

Summary of post-mortem findings

(1) Carcinoma in substance of the pancreas with hepatic and glandular metastases.

(2) Bilateral adreno-cortical hyperplasia.

(3) Fuchsinophil (androgenic) granules of Vines in epithelium of both tumour and adrenal cortex.

(4) Anterior pituitary: basophilia (relative increase in basophil cells), extreme basophil-cell hyalinization, three chromophobe adenomata.

DISCUSSION

The tumour in the pancreas

The tumour has the structure of a *malignant adreno-cortical adenoma*; fuchsinophil granules are present, a common finding in the adreno-cortical tumours associated with masculinization [Broster & Vines, 1938]. The tumour is present in a site where adreno-cortical rests have been shown to occur [Ribbert, quoted by Glynn, 1911] and is associated with a group of symptoms similar to those commonly present in cases with adreno-cortical neoplasm. Both macroscopically and microscopically the tumour is shown to have no direct connexion with the adrenal glands. Accordingly, it is an adenocarcinoma of an adreno-cortical rest. Hyperplasia or tumour in adrenal rests is uncommon and their association with sexual alteration is exceedingly rare. Glynn [1911], in a review of the literature, quoted three cases of pseudohermaphroditism associated with large adrenal rests. Since then Kolodny [1934] has reported what he considers to be a unique example in an adult female with a clinical picture similar to the present case but with more definite hypertension and with enlargement of the clitoris. This was due to a carcinoma of an adreno-cortical rest situated in the neighbourhood of the solar plexus and removed by operation. The patient improved greatly after the operation but ultimately died with pulmonary metastases. The findings were not verified by autopsy. Some masculinizing tumours of the ovary take origin from ovarian adreno-cortical rests [Novak, 1941].

Taking into consideration the short course of the disease in this case, it seems likely that the primary condition is the carcinoma of the cortical rest. We consider that the bilateral adreno-cortical hyperplasia is produced by the same stimulus (unknown) which induced malignant change in the adreno-cortical rest. Reasons will be given later for assuming that the hypophyseal changes are secondary to the adreno-cortical abnormality. Grollman [1936] has propounded the theory that hyperplasia or tumour of the adrenal cortex associated with masculinization is produced from a special juxta-medullary zone of the adrenal cortex. We have not found any indication from the morphology of the enlarged adrenals and the adreno-cortical tumour to indicate the implication of such a tissue in the morbid process of the present case.

The hypophyseal abnormality

Basophil-cell hyalinization. Crooke [1935] has shown that a conspicuous degree of basophil-cell hyalinization is the only constant lesion in, and is specific for, Cushing's syndrome whether there be a tumour of the basophil cells, of the adrenal cortex, or of the thymus, and all subsequent evidence appears to substantiate this view [McLetchie, 1944*b*]. In our case the hyalinization is greater in degree than has ever been previously recorded; this finding is possibly in parallel with the large amount of abnormal adreno-cortical tissue both as tumour and cortical hyperplasia. Crooke specially noted that, despite the replacement of the basophil granules by the refractile hyaline cytoplasm, the nucleus of the hyaline basophil cell showed no abnormality. The apparent normality of the nucleus is a striking feature in our case despite the remarkable degree to which hyalinization has proceeded so that the nuclei appear embalmed in a dense wax-like cytoplasm. Since the nucleus was normal Crooke concluded that basophil-cell hyalinization was an attribute of some special alteration in basophil-cell

activity rather than a stage to cell death. In our case the derangement of basophil-cell activity is of extreme degree and it cannot be denied that the accepted morbid basis of Cushing's syndrome is present.

Basophilia. Basophilia has been previously recorded in a case of adreno-cortical tumour associated with Cushing's syndrome [Lescher & Robb Smith, 1935]. Basophil-cell hyalinization was also present in this case [Crooke, 1935]. In connexion with the importance of this finding, the active development of basophil cells from the pars intermedia and associated epithelial inclusions found in the posterior lobe in our case is of importance. While columns of wandering basophil cells in the posterior lobe are often found to be continuous with the epithelium of the pars intermedia, we have not observed in our control series [cf. McLetchie, 1944*b*] active differentiation of basophil cells from large numbers of pars intermedia cells as obtains in the present case. Lewis & Lee [1927] have already drawn attention to clefts in the posterior lobe lined with epithelial cells similar to our finding. We have also encountered these structures in our control series but unassociated with production of basophil cells. We thus consider that the active development of basophil cells from the pars intermedia and allied tissues is associated with the general basophilia and is evidence of a stimulus for increased basophil-cell activity which is part of the morbid process.

The chromophobe adenomata. Chromophobe adenomata have been recorded in cases with a similar pathology to our own, but associated with a symptomatology more obviously belonging to Cushing's syndrome [McCullagh & Cuyler, 1937; Graef *et al.* 1936]. Close [1934] records the finding of chromophobe adenomata in 10% of pituitary glands from routine autopsies. He does not distinguish between true chromophobe and 'indeterminate' adenomata. Some adenomata which we have encountered in routine autopsy material have been 'indeterminate' in character and it is probable that Close's series contained examples of this type. Graef *et al.* have pointed out that the so-called chromophobe adenomata are rare in young subjects, and they consider that the adenoma in their case is associated with the morbid process and is not an incidental finding. Since, however, these adenomata are sometimes found unassociated with adrenal tumour, and are not constantly associated with adrenal tumours, it would appear that they can have only some secondary significance in relation to the morbid process under discussion. Cramer & Horning [1936] and others have produced large chromophobe adenomata in the hypophyses of rats by injections of oestrogen. The chromophobe adenomata found in cases of adrenal tumour may be correlated with this experimental finding, in that large amounts of oestrogen have been shown to be excreted in such cases [Frank, 1934; Saphir & Parker, 1936]. In Graef's case the oestrogen excretion exceeded that of pregnancy.

The relationship of the clinical to the morbid anatomical findings

The comatose state followed by sudden death was apparently due to hepatic failure, coma and delirium being not uncommonly associated with massive necrotic secondary carcinoma of the liver, though this sudden termination preceded by convulsions is unusual and the possibility that the coma was due to the extreme basophil-cell changes cannot be excluded.

At no time did the patient's condition suggest that a morbid process was at work as extensive as we have found, and on clinical grounds the patient's condition might be

regarded as one of virilism showing only coarsening of the features, hirsuties, amenorrhoea, slight hypertension, and dusky cyanosis. The symptomatic history of the disease was, however, only of 12 weeks' duration and our view is that had the patient lived longer more obvious signs of Cushing's syndrome would have developed as a result of the specific pituitary change. With regard to the development of Cushing's syndrome in cases of adreno-cortical tumour, it is of interest to consider the course of two cases pathologically similar to ours.

Hare, Ross & Crooke [1935] reported the case of a female aged 31 years in whom the first significant clinical signs were changes in facial appearance, with obesity and the growth of a beard; one year later amenorrhoea followed a period of metrorrhagia. Later, a full Cushing's syndrome developed and the patient died after a surgical operation for removal of an adreno-cortical carcinoma. Post-mortem examination showed hyalinization of the basophil cells. This clinical picture of initial virilism followed by Cushing's syndrome was also shown by the patient reported by Lescher & Robb Smith [1935]. In this case, in a female aged 32 years, menstruation ceased abruptly, obesity appeared and was followed by the growth of a beard and moustache. When examined three years after the onset of the disease, she presented the typical appearance of Cushing's syndrome with obesity, hirsuties, plethoric appearance, dusky cyanosis, hyperglycaemia, polycythaemia, hypertension, and marked osteoporosis. As in the previous case, death occurred after the removal of an adreno-cortical carcinoma and again hyalinization of the basophil cells was found at autopsy [Crooke, 1935]. It would appear that the present case differs from these mainly in its short duration and only the extreme malignancy of the tumour prevented a similar course being taken. The three cases are presented as illustrating a possible sequence in the genesis of the Cushing's syndrome.

On general principles, it is most unlikely that such a profound derangement of basophil-cell function as is manifested by the extreme hyalinization in our case would only be associated with manifestations of virilism. On the other hand, it cannot be doubted that hypercortico-adrenal conditions of long duration may be only associated with manifestations of virilism without developing evidence of multi-glandular disease, though this is more true of cases with adreno-cortical hyperplasia than with tumour [see Broster & Vines, 1938].

Broster & Vines have made the widest study of adrenal virilism (the adreno-genital syndrome) and have shown that in the adolescent female, as the result of adreno-cortical lesions, more or less complete sex reversal may occur, but in the adult female the capacity for sex reversal is largely lost through loss of plasticity of the tissues. In the latter instance the only abnormalities usually found are those of hirsutism, amenorrhoea or irregular menstruation, slight deviation of the body contours towards the male type, and sometimes distressing psychological changes.

It would thus appear that a hypercortico-adrenal condition may produce either a condition of virilism or may lead to the multiglandular disturbance of Cushing's syndrome. It is of importance to note that Crooke did not find basophil-cell hyalinization in a case of adreno-cortical tumour with virilism unassociated with any of the additional signs of Cushing's syndrome. He considers that the production of basophil hyalinization is the factor which determines the development of Cushing's syndrome, while, presumably, virilism is the result of hypercortico-adrenal stimulation uncompli-

cated by altered activity of the basophil cells. If this is so, one would expect to find differences in the adrenal abnormalities of the two conditions. On qualitative grounds, we know of no difference between the adrenal lesions; for instance the fuchsinophil granules of Vines, a constant finding in adrenal virilism, are also present in adrenal tumours associated with a fully developed Cushing's syndrome [Hare *et al.* 1935; Lescher & Robb Smith, 1935]. On the other hand, a quantitative difference exists in that, while adreno-cortical hyperplasia is commonly associated with virilism, cases of adreno-cortical tumour usually rapidly develop manifestations of multiglandular upset [Broster & Vines, 1938]. This suggests that the difference between adrenal virilism and Cushing's syndrome associated with an adrenal tumour is one of degree, the basophil-cell abnormality necessary for Cushing's syndrome only being produced by hypercortico-adrenal conditions of high degree.

McLetchie [1944*b*] considers that in Cushing's syndrome associated with adrenal tumour the sequence of events is as follows: the adreno-cortical hyperactivity results in basophil-cell overactivity which may be associated with basophil-cell increase (basophilia); basophil-cell hyalinization is a cytoplasmic change consequent on basophil-cell overactivity.

It is evident from our case that a profound basophil-cell response may be rapidly elicited in the anterior pituitary by a hyperadreno-cortical condition without much external evidence, and it is apparent from Lescher's and Hare's cases that it takes time for unequivocal signs of multiglandular upset to develop.

Up to this point we have passed over the possibility of the hypophyseal changes in our case being antecedent to the adreno-cortical abnormality. This would be unlikely if return to normal could be obtained after removal of an adreno-cortical tumour in a case where the symptomatology left no doubt that the morbid process had passed beyond that of pure hypercortico-adrenal stimulation. Unfortunately, no case with the full clinical picture of Cushing's syndrome has survived operative removal of the tumour. Walters, Wilder & Kepler [1934] have recorded a series of cases under the heading of 'The Adreno-Cortical Syndrome' in at least one of which complete return to normal was obtained after removal of an adrenal tumour. The symptomatology of their cases is much more suggestive of early Cushing's syndrome than of uncomplicated hypercortico-adrenal stimulation and we consider that one must infer that the basophil-cell alterations of Crooke can be secondary to an adrenal tumour and are reversible.

SUMMARY

A case is described of the sudden development of a condition of mild virilism in a previously healthy and apparently normal adult female. Sudden death followed a brief period of coma three months after the onset of the first signs. Post-mortem examination showed a carcinoma of an adreno-cortical rest and bilateral adreno-cortical hyperplasia. In the anterior pituitary gland extreme basophil-cell hyalinization, basophilia, and three chromophobe adenomata were found.

The relationship of adreno-cortical hyperplasia and tumour to the anterior pituitary changes is discussed.

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DISTURBANCES OF WATER BALANCE IN THE RAT ON REMOVAL OF THE ADRENAL MEDULLA

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In an investigation on hair loss in medullectomized animals [Stein & Wertheimer, 1941] skin disturbances which suggested some interference in the water metabolism were noted. Changes in water balance, related in part to disturbances in the mineral balance, are well known after adrenalectomy. The question whether absence of the adrenal medulla contributes to the disturbance of the water balance remained open. In the present investigation the water balance of medullectomized rats is investigated by (1) water diuresis tests, (2) water intoxication experiments, and (3) normal drinking tests.

ANIMAL MATERIAL

Male albino rats of laboratory rearing were employed for the experiments. Their average weight was 200 g. (160–250 g.).

METHOD OF MEDULLECTOMY

Medullectomy was effected by the methods of Evans [1935] and Ingle & Higgins [1938] under ether narcosis. In a small number of cases, the adrenal capsule was torn open with a fine-toothed forceps and then bluntly enucleated. In sham-operated rats, both adrenals were pulled forward and the adrenals cut free of surrounding adipose tissue as far as the blood vessels.

NUTRITION

Operated and control animals were maintained on tap water and on a mixture of cereals and vegetables. In the winter of 1941–2 the diet consisted either of maize and vegetables or of a synthetic feeding mixture composed of 20 % casein, 70 % potato meal, 10 % olive oil, 2 % salt mixture, 3 % dry yeast, and 25 drops of cod-liver oil.

BEHAVIOUR OF MEDULLECTOMIZED RATS

For some time after operation the rats showed latent cortical deficiency. Experiments were therefore performed to determine the time after medullectomy when cortical function was restored sufficiently to ensure absence of disturbances due to cortical deficiency. This was determined by several methods.

(*a*) Medullectomized, sham-operated, normal, and adrenalectomized rats were exposed to a temperature of about $-7°$ C. for 4 hr. after fasting for 15 hr. Up to the fifth post-operative day, two-thirds of the medullectomized rats showed an abnormal fall in temperature when compared with the sham-operated and normal animals, but only 16 % suffered temperature shock comparable with that regularly induced in the adrenalectomized animals. Between 6 and 10 days after operation, 40 % of the animals showed an abnormal fall in temperature, but in every case the

degree of disturbance was less than in the adrenalectomized rats. Between 11 and 28 days after operation, the average falls in temperature in the medullectomized and normal animals were equal. Marked temperature decreases were observed in this period only in exceptional cases.

(*b*) The length of life of the animals was not affected by medullectomy, and addition of common salt to the diet to prevent insufficiency symptoms was never necessary. Of a number of medullectomized animals kept under observation for longer than a year, only a few died of pneumonia. Decrease in the amount of sodium and increase in the amount of potassium fed did not affect the mortality rate.

(*c*) Specific reactions to hunger such as are observed in adrenalectomy were not evinced by the medullectomized rats. The carbohydrate metabolism (blood sugar, liver glycogen, and muscle glycogen) was not affected.

(*d*) All medullectomized animals showed satisfactory regeneration at autopsy. Histological examination of ten regenerated specimens showed: medulla—absent in all cases; zona glomerulosa—present, but in two cases irregular and in places absent; zona fasciculata—uniformly present and always well developed; zona reticularis—absent in all cases.

WATER DIURESIS EXPERIMENTS

Method

The tests were carried out by the method of Heller & Urban [1935]. Groups of three rats of equal weight were fasted overnight (16–17 hr.) on tap water, or, in a small number of cases, on Rubin-Krieck solution. In the morning tap water at 30° C. was administered by stomach tube in a dose of 5% of the body weight before fasting. The urine was collected in 15 min. lots in Burn diuresis cages [1937; cf. Truszkowski, Blauth-Opicuska & Jwanowska, 1939]. The times in which 50% of the quantity of water ingested was excreted and the total amounts of urine excreted in 3 hr. were determined.

Results

Normal rats. Normal diuresis after water administration by stomach tube was determined in forty-five experiments involving thirty-three groups of rats. 50% excretion (maximum excretion time) was found at 108 min. (71–155 min.). The excretion after 3 hr. amounted to 68·4% (51·5–92·1%) of the amount of water ingested. The season of the year did not seem to affect the results, the maximum excretion time in April–September 1941 being 110 min. and the corresponding figure for November–March 1942 114 min. Temperature and air humidity were also without effect on the time course of the excretion curve. Sham-operated animals behaved practically normally. Excretion set in after a period of anuria of 30 min. on the average and followed a characteristic course (Fig. 1, curve 1).

Medullectomized rats. Twenty groups of animals were examined. The averages for ninety-five experiments during the period March 1941–August 1942 are seen in Table I (see also Fig. 1, curve 2).

Water excretion in the first week after operation was strongly inhibited, but rarely to an extent equalling that after adrenalectomy. The urinary excretion in 180 min. later fluctuated between 10 and 30% of the ingested water as against 68·4% in normal animals, but was very constant within any given group. After 3–4 weeks

an improvement in the diuresis figures to an average value equal to the minimum normal excretion (Fig. 2) was seen. It is noteworthy, however, that this improvement was not maintained in later experiments. Out of sixteen groups which were tested 91–290 days after operation, 40 % showed a 50 % excretion time of over 180 min. (max. 260 min.) as against 108 min. in normal animals.

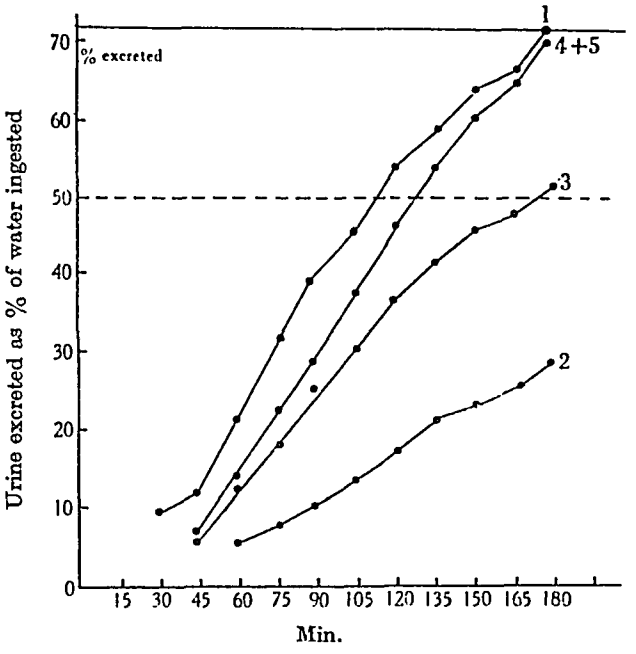


FIG. 1. Urine excretion in water diuresis tests. Average of all experiments up to March 1942. Curve 1: controls. Curve 2: medullectomized rats 11–25 days after operation. Curve 3: medullectomized rats 26 days after operation. Curve 4: medullectomized rats less than 26 days after operation injected with adrenaline. Curve 5: adrenalectomized rats injected with adrenaline.

Table 1. *Water diuresis test in medullectomized rats*

	Days after operation				Normal rats
	5–10	11–25	26–90	90–290	
50 % excretion time in min.	> 300	> 300	147	174	108
Urine excreted in 3 hr. as % of water ingested	13·2	25·6	51·1	47·6	68·4

The initial anuria, which lasted 30 min. in the control animals, lasted 60 min. on the average in medullectomized rats 4–10 days after operation, and 50 min. 11–25 days after operation. The curve of urinary excretion in medullectomized and normal rats after a single administration of water by stomach tube is shown in Fig. 1. The curve is based on averages calculated from the total data gathered up to March 1942. In the first 3–4 weeks after operation the water excretion curve is flatter in the medullectomized rats than in the controls. 3 hr. excretions amounting to 50 % of the total water ingested, which is the normal minimum figure, were only observed in medullectomized rats more than 3–4 weeks after operation. The experiments could be reproduced with little relative variation at all seasons of the year, except during April–July 1941 and 1942. During April–July the inhibition of diuresis was already

diminished after the 14th post-operative day. In all medullectomized animals diuresis only improved to the value of the minimum normal excretion; the normal average was not attained (Figs. 1, 2). Diuresis was not influenced by repetition of the water test. Administration of water by stomach tube did not exert a deleterious influence, and neither the weight nor the body temperature of the medullectomized animals was influenced by this treatment.

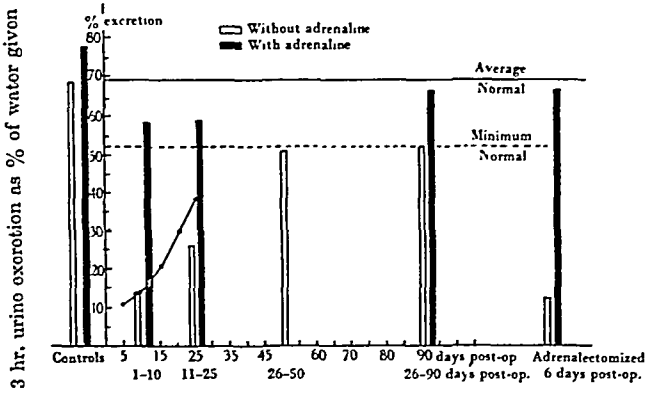


FIG. 2. Urine excretion in 3 hr. after water ingestion in control, medullectomized and adrenalectomized rats with and without adrenaline injection.

Effect of adrenaline on water diuresis

l-Adrenaline supplied by Poulenc Frères or by Assia was used. The animals received 0·1 mg./100 g. body weight subcutaneously in 0·2 ml. of distilled water 30 min. before water ingestion. The influence of the treatment on the water excretion at different times after medullectomy is seen in Figs. 1 and 2. Sham-operated and normal control animals so injected showed only a small change or none at all in the values for total excretion. No effect at all was demonstrable on the time of 50% excretion. In medullectomized animals, on the other hand, a definite increase and acceleration of diuresis was produced by adrenaline (Figs. 1, 2, and Table 2). A very notable effect was produced in the adrenalectomized animals. Prior to and during the experiments the rats were in good condition; body weight and temperature were not affected by the operative treatment. Animals in the adrenalectomized group received Rubin-Krieck solution until the start of the test.

Table 2. *3 hr. urine excretions in percentage of ingested water after injection of adrenaline in a dose of* 0·1 *mg./*100 *g. of body weight*

(Values for untreated animals are given in brackets)

	Days after operation		
	5–10	11–25	11–73
Medullectomized rats	57·4 (13·2)	57·2 (25·6)	64·1 (51·1)
Adrenalectomized rats	66·2 (4·9)	—	—
Normal rats	78·0 (66·5)	—	—

Whereas untreated adrenalectomized rats excreted little or no urine in the first 3 hr. after water ingestion, the adrenaline-injected adrenalectomized rats showed a prompt and sustained diuresis which occasionally even exceeded that evinced by medullectomized rats at the same post-operative stage. Adrenaline treatment is seen to curtail the initial period of anuria in control and medullectomized animals to 15 min. The majority of medullectomized rats and a number of the controls were markedly restless following adrenaline injection. Frequently the animals stretched full length on the cage floor, sometimes lying prone only to leap suddenly to their feet after a minute or two, and then repeated the process. The excretion of the highly concentrated initial sample of urine was visibly difficult. Respiration at this time was strikingly deep and regular; the peripheral circulation was undisturbed. After the onset of diuresis the animals became quieter.

Minimum effective dose and duration of activity of adrenaline

30 μg. of adrenaline injected subcutaneously 30 min. before water ingestion by stomach tube were sufficient to induce optimal diuresis (Table 3).

Table 3. *Different doses of adrenaline* 30 *min. before water ingestion*

Dose μg.	No. of exps.	50% excretion in min.	Excretion in 180 min. %	Excretion of chlorine mg.
5	2	> 300	40·8	Traces
10	5	> 300	21·6	Traces
30	2	145	96·0	18·8

The adrenaline effect sets in 30 min. after injection and disappears after 5 hr. (Table 4).

Table 4. *Duration of effect of adrenaline in a dose of* 100 μg./100 *g. body weight administered subcutaneously*

Interval between adrenaline injection and water ingestion min.	50% excretion min.	Excretion after 180 min. %	Excretion of Cl mg.
0	110	104	34·0
30	67	129	60·9
120	45	107	29·1
180	135	51	11·6
240	75	81	16·9
300	> 300	11	Traces

Influence of adrenaline on chlorine excretion

It is apparent (Table 5) that adrenaline injection increased the chlorine excretion during the diuresis test even in normal rats, but a very much greater increase was observed in medullectomized rats during the time when diuresis was inhibited.

Adrenalectomized rats. Adrenalectomized rats which had been maintained on Rubin-Krieck solution for many days before the water diuresis test excreted 30 mg. of chlorine during the first 180 min. of the test when they were injected with 100 μg. of adrenaline per 100 g. body weight. This high excretion is due to the Rubin-Krieck solution, since normal rats receiving this solution also excreted large amounts of

Table 5. *Average chlorine excretions in 3 hr. in water diuresis tests*

	Excretion of chlorine mg.
(*a*) Normal rats	
Untreated	1·3
After adrenaline injection, 0·1 mg./100 g. body weight	10·8
(*b*) Medullectomized rats	
Untreated	Traces
After adrenaline injection	
5th–10th day after operation	14·6
11th–25th day after operation	23·1
26th and more days after operation	10·0

chlorine in the test (32 mg.). Adrenalectomized rats which did not receive an injection of adrenaline showed complete inhibition of diuresis and their chlorine excretion was therefore nil.

Influence of adrenaline-like substances on water diuresis in medullectomized rats

Subcutaneous injections of tyramine (10 mg.), ephetonine or ephedrine (7, 10 and 100 mg.), and of strychnine, caffeine, cardiazol, or atropine in different doses had no effect on the inhibition of water diuresis exhibited by medullectomized rats. Benzidrine (2·5 mg.) given by mouth was also ineffective.

Influence of desoxycorticosterone

5 mg. of desoxycorticosterone acetate were administered per 100 g. of body weight 6 hr. before water ingestion. Four experiments giving similar results showed that desoxycorticosterone acetate abolishes the inhibition of diuresis in the water ingestion test, accelerates the 50% excretion, and is without effect on the chlorine excretion. The active dose is very large. Smaller doses were entirely ineffective (Table 6). Feeding of Rubin-Krieck solution during 3 days before the actual experiment with or without subcutaneous injections of salt solution did not affect the inhibition of diuresis in the medullectomized rats.

Table 6. *Water diuresis tests on medullectomized rats after treatment with desoxycorticosterone acetate*

	Untreated	Treated
(*a*) Dose of 5 mg./100 g.		
50% excretion time (min.)	>300	85
Excretion after 3 hr.	13·5%	67·7%
(*b*) Dose of 3 mg./100 g.		
50% excretion time (min.)	>300	>300
Excretion after 3 hr.	17·7%	15·1%

WATER INTOXICATION IN MEDULLECTOMIZED RATS

Methods

Water intoxication was produced by the method of Gaunt, Remington & Schweizer [1937]. After a 12-hr. fast the animals received tap water at 40°C. in 5-hourly administrations by stomach tube. Two experiments were run; in one the animals

received 6 ml./100 g. of body weight and in the second 9 ml./100 g. of body weight as determined after fasting. The rats were weighed at the start of the experiment, and 6 and 24 hr. later. The amount of fluid excreted was measured hourly until 1 hr. after the last water administration; total excretion was measured after 24 hr. The excretion was made up of (*a*) pure urine, (*b*) urine mixed with intestinal fluid, and (*c*) pure intestinal fluid. In order to determine the influence of shock, pulse rate and body temperature were recorded 6 and 24 hr. after the first water treatment, the pulse rate being taken by a count of the heart apex beat in so far as this was feasible.

Results

Table 7 shows the average values for medullectomized and control animals.

Table 7. *Water intoxication in medullectomized rats*

Animals	No. of exps.	No. of deaths	% fluid excreted in 6 hr.	% fluid excreted in 24 hr.	Temp. drop at 6 hr. °C.	Pulse at 6 hr.
(*a*) 9 ml. of water per 100 g. body weight						
Controls	8	0	51·5	76·5	− 2·7	500/min. and more
Controls + adrenaline*	4	0	62·3	87·9	− 2·0	600/min. and more
Medullectomized 20–250 days post-op.	12†	12	29·3	—	−10·4	230/min.
	3†	0	49·8	90·0	—	
Medullectomized 6–316 days post-op. + adrenaline*	11	3‡	43·0	60·8	− 4·5	450/min. and more
(*b*) 6 ml. of water per 100 g. body weight						
Controls	18	0	63·3	73·3	− 0·9	500/min. and more
Medullectomized 9–18 days post-op.	6	6	7·5	—	− 9·8	Less than 230/min.
Medullectomized 22–284 days post-op.	24	1	39·6	65·0	− 2·5	450/min. and more

* Dose of adrenaline was 0·15 mg./100 g.

† Out of fifteen animals investigated twelve behaved pathologically and three were found to be normal.

‡ The three rats which succumbed (5½, 6½ and 48 hr. after the first water ingestion) showed symptoms of shock.

Behaviour of normal animals

No rats were killed by either dose of water. Out of eighteen control animals which were given the smaller dose only two showed a disposition to cramps (sudden twitching when tapped), while of eight control rats which received the larger dose one developed cramps and two showed disposition to cramps.

With the dose of 6 ml./100 g. body weight, sixteen out of the eighteen control animals excreted pure urine only. In two cases both urine and intestinal fluid were excreted during the fourth and fifth hour after the first water ingestion. Fig. 3 shows that diuresis set in within the first hour, that the urinary excretion continues to increase (in comparison with the amount of water ingested) up to the last hour but one, the two final portions being equal (maximum).

With the larger dose of water (9 ml./100 g. body weight), three out of eight rats excreted pure urine, two excreted intestinal fluid as well as urine in the last hours only, two excreted both intestinal fluid and urine throughout the observation period, and one excreted intestinal fluid alone. Diuresis set in within an hour of the first water ingestion and increased steadily.

Behaviour of medullectomized rats

Small dose of water. Up to the 18th day after operation, all the treated animals died on an average 7 hr. after the first water ingestion. All the animals developed oedema particularly in the eye regions and in the limbs. Two rats showed disposition to cramps, one died of cramps, and five died without cramp symptoms. At autopsy, cortical regeneration was found in all the animals. Water excretion was in every case inhibited. Two animals failed altogether to excrete, two excreted pure urine, and two both urine and intestinal fluid. It is noteworthy that at autopsy the bladder in most rats was found fully distended (shock).

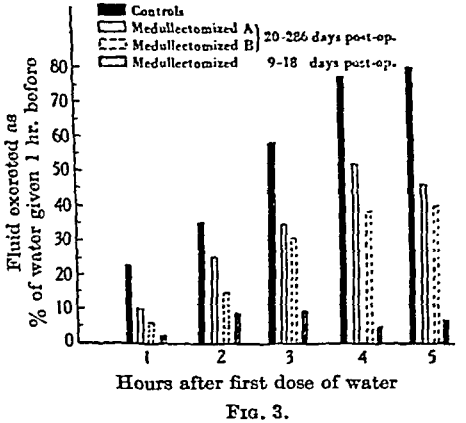


Fig. 3.

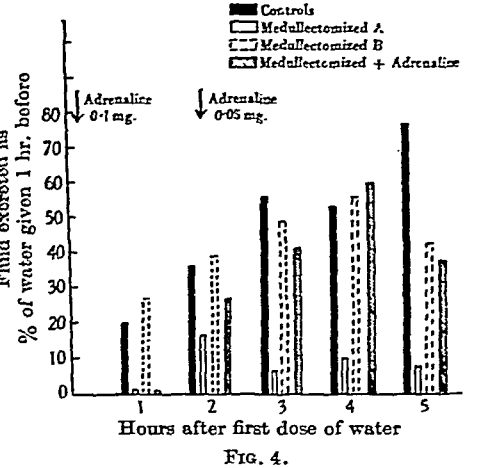


Fig. 4.

Fig. 3. Fluid excretion in water intoxication experiments (6 ml. of water per 100 g. body weight). Height of each column represents the fluid excretion expressed as a percentage of the water given 1 hr. previously.

Fig. 4. Fluid excretion in water intoxication experiments (9 ml. of water per 100 g. body weight). Height of each column represents the fluid excretion expressed as a percentage of the water given 1 hr. previously.

Externally the rats medullectomized 22–284 days previously showed little difference from the normal group. Only one rat (31 days after operation) of the total died in cramps.

Fourteen animals excreted urine only (*A* in Fig. 3); ten excreted intestinal fluid only (*B* in Fig. 3). Fig. 3 shows that the delayed effect of medulla deficiency is one of slowed water excretion. The normal curve rises steeply in the first 6 hr. and then straightens out with little subsequent change. The ascent of the curve in the case of medullectomized rats is far less steep, and reaches the maximum excretion value of the normal animals only after 24 hr.

Large dose of water. Three of the animals behaved practically normally. They are specified in Table 7. Twelve other medullectomized rats died with cramps and severe oedema on an average 6 hr. after the first dose of water.

Four rats excreted pure urine only (*A* in Fig. 4). Their diuresis only set in during the second hour and remained rather low. In eight rats, intestinal fluid only was excreted (*B* in Fig. 4). In their case excretion set in only in the second hour and all

the animals died, even though until the fourth water treatment the amount of fluid excreted by them through the intestine was equal to that excreted by controls through the kidneys (Fig. 4).

Effect of adrenaline. Adrenaline was administered in dosage of 0·1 mg./100 g. body weight with the first dose of water and 0·05 mg./100 g. body weight with the second dose.

Control animals. All animals in this group excreted urine at an accelerated rate, and excreted urine only.

Medullectomized rats. Out of eleven animals in this group only three died (tested 39, 96 and 250 days after operation respectively), one in the first hour and the other two after 6 hr. Six animals excreted urine only; four excreted intestinal fluid as well as urine; one excreted intestinal fluid only. Excretion began in the second hour and then rose in a course parallel to that of the controls but in the fifth hour was decreased again. The 24 hr. value was lower than in the control group.

Desoxycorticosterone acetate was administered in a total dose of 12–21 mg. per rat in three to four injections given over a period of 24 hr. The last injection was given 4 hr. before the first water administration. One of the animals behaved normally, two died 6 and 30 hr. from the beginning of the experiment, and one suffered shock but later recovered.

NORMAL FLUID METABOLISM OF MEDULLECTOMIZED RATS

Male rats of about 200 g. body weight were maintained for 1 week in metabolism cages. The separated urines were collected and the amount of water ingested was measured. Animals which failed to learn to drink from the tube of the water flask within a short time were eliminated from the test group. For most of the experiments a synthetic diet was supplied; in the early summer of 1941 maize meal was given; in spring 1942 wheat and vegetables were used. Animals on the latter diet showed no differences from animals on synthetic diet. All values given are averages calculated per 100 g. body weight. Medullectomized rats were investigated at varying times after operation. Three series of experiments were performed: the first in summer 1941, the second in winter 1941–2, the third in summer 1942.

Controls

The averages for water ingestion and urine excretion by the control animals are presented in Table 8.

Similar amounts of urine were excreted in the two summer experiments. Twice as much was excreted in the winter experiment.

Similar amounts of water were ingested in the experiments conducted in the first two half-year periods. The ingestion of water in the experiment performed in the second summer was somewhat higher. Sham-operated rats showed the same behaviour as the normal animals and are reported together with the latter in the table. Despite daily fluctuations in the ingestion and excretion of water a condition of equilibrium is clearly discernible in the controls. In no normal case was there a delayed rise in the water intake or urine output or a sustained maintenance of the increased values, apart from the increased water ingestion and diuresis observed during dry hot weather (khamsin) in spring; the abnormality disappeared, however, immediately after a change in the weather.

Table 8. *Fluid balance*

	No. of exps.	No. of animal	ml. of water ingested per 100 g. body weight per day: Average	Days post-op. 2–30	30–92	93–365	ml. of urine excreted per 100 g. body weight per day: Average	Days post-op. 2–30	30–92	93–365
				Summer 1941						
Medullectomized	47	22	6·9	6·3	7·6	—	2·4	1·67	2·9	
Controls	20	19	5·1				1·03			
				Summer 1942						
Medullectomized	30	21	12·3	16·0	11·3	8·8	4·9	7·1	4·2	3·0
	16	16	8·8				3·6			
Controls	10	10	6·6				1·4			
	6	6	6·6				1·5			
				Winter 1941–2						
Medullectomized	15	15	6·0				2·1			
Controls	11	11	5·35				2·18			
				Results of all summer experiments						
Medullectomized	93	59	9·3				3·6			
Controls	36	35	6·1				1·3			

Medullectomized rats. Summer experiments

The water balance of medullectomized rats is disturbed as compared with controls and distinguished by an increase both of ingestion and excretion. Certain of the medullectomized rats yielded values which may still be regarded as falling within the normal range, but in 70% of all the medullectomized cases the fluid intake and output was definitely increased. This disturbance of the water balance was sustained.

Behaviour in the first post-operative month. In the first month after operation increased water ingestion and enhanced diuresis were encountered relatively rarely and mostly in a relatively mild form. Rats operated in spring (April–June 1942) were exceptional in this respect, and yielded unusually high values. In this season cases of disturbance in the water balance due to the adrenal cortex were encountered. These cases are easily diagnosed and do not occur later than the third or fourth day after operation.

Behaviour in the second and third post-operative months. In this period pronounced summer polydypsia and polyuria were observed. All the experimental animals remained in good condition. Ingestion of food by the medullectomized rats was equal to that of the controls.

Behaviour in fourth to twelfth post-operative months. The animal material available at this period was restricted, as some of the operated adult rats had succumbed after several months to pneumonia. The majority of surviving animals showed sustained disturbance of the water balance but in milder degree than in the preceding period (Tables 8, 9).

Experiments performed in winter 1941–2

In the experimental series run in winter no difference between medullectomized and control animals in regard to amounts of water ingested or excreted were discerned.

THIRST EXPERIMENTS

Denial of water for a period of 24 hr. affects medullectomized and control rats equally. The amount of urine excreted amounted in both cases to one-quarter of that recorded for the period before the withdrawal of the water supply. In consequence, excretion by thirsting medullectomized rats is higher than in thirsting controls. On restoring the water supply, the excretion values reverted to their pre-thirst level in both cases within a day. Drinking tests were conducted further on fasted rats with a view to concurrent determination of the chlorine excretion. No difference in chlorine excretion between medullectomized and normal rats could be found. The medullectomized rats were tested 31–81 days after operation. The result of drinking tests on rats in a 15-hr. fast period did not deviate from findings on non-fasted animals. In both cases, the medullectomized rats displayed polydypsia and polyuria (see Table 10). After a 24-hr. fast, a difference between operated animals and controls was no longer discernible. This is due to the circumstance that in normal animals so long fasted hunger polyuria sets in, whereas in medullectomized animals (eight experiments on animals 76–95 days after operation) this development fails to occur.

Table 9. *Late post-operative behaviour of medullectomized rats*

Date of operation	Days after operation	ml. of water ingested per 100 g. body weight	ml. of urine excreted per 100 g. body weight	Observation period
22. i. 42	43– 48	4·8	2·6	March 1942
	109–118	7·8	1·8	May 1942
	152–157	14·1	6·8	June 1942
	158–164	14·4	6·6	June 1942
	165–173	14·6	8·0	July 1942
30. v. 41	59– 65	7·3	2·8	Summer 1941
	91– 92	7·5	3·4	Summer 1941
	241–249	5·6	1·7	January 1942
	346–354	8·4	1·4	May 1942
	378–387	8·7	2·8	June 1942

Table 10. *Drinking tests during fasting*

Animals	No. of groups	No. of exps.	ml. of water ingested	ml. of urine excreted	mg. of chlorine in urine
Medullectomized	4	11	39·0	24·2	11·0
Controls	4	9	21·0	12·2	15·0

The values here tabulated are not referred to 100 g. body weight but are reported as per animal.

DISCUSSION

Medullectomized rats several days after operation evince none of the symptoms characteristic of adrenal cortical deficiency. They subsist on ordinary diet for the normal life span. In susceptibility to cold or hunger they are not abnormal and they show no known symptoms of disturbance in carbohydrate or mineral metabolism. Water diuresis tests demonstrated serious disturbance of the water excretion in medullectomized rats during the first 4 weeks after operation. After this time their

diuresis undergoes some improvement but does not surpass the minimum value of normal animals under like experimental conditions; after a long delay, indeed, a relapse of the excretory condition is often observed. The shape of the excretion curve deviates markedly from the normal in the early post-operative stage and still deviates appreciably in the later stage.

Adrenaline can lessen and even abolish the inhibition of diuresis in the medullectomized and adrenalectomized animals. Desoxycorticosterone acetate abolishes the inhibition in the medullectomized animals only in the relatively high dose of 5 mg./100 g. body weight. Adrenaline accelerates chlorine excretion even in normal animals, but does so much more markedly in medullectomized rats, while water diuresis is inhibited. Desoxycorticosterone acetate does not influence the chlorine excretion.

The medullectomized animals showed, also, markedly enhanced susceptibility to water intoxication. In the first post-operative month, both the smaller and the larger water doses given were lethal to the medullectomized rats, whereas the normal animals generally survived. In the medullectomized group the treatment caused oedema, cramps, and severe falls in body temperature and pulse rate, but these symptoms were either altogether absent or only present in a very mild degree in the normal group. In the medullectomized group in the first post-operative month the time of water excretion was markedly lengthened. Excretion of intestinal fluid as well as urine was frequently encountered. At periods later than 30 days after operation the difference between medullectomized and normal animals in response to the lower dose of water was limited to an increase in excretion time. The condition of the water-intoxicated medullectomized rats could be improved markedly by adrenaline administration. Desoxycorticosterone acetate on the contrary was of questionable influence.

Data on the fluid balance of medullectomized and normal rats under physiological conditions have been given. The medullectomized animals during the first post-operative month showed polydypsia and polyuria in a mild form which became increasingly pronounced in the second and third months, and later was long sustained in a moderate form. These symptoms were surprisingly not encountered in winter. They were not affected by adrenaline administration, and in general they appear to be of a different nature from the disturbances hitherto mentioned. They become marked for example only in the second month, whereas the disturbances earlier mentioned were already most pronounced in the first month. Moreover, unlike the previously mentioned disturbance they are conditioned by the seasons. The causes of this 'diabetes insipidus' in medullectomized rats remain obscure.

The genesis of the pathological reactions shown by medullectomized rats during water diuresis and in the water intoxication test remains to be considered. The noted disturbances in water balance cannot be connected with disturbances in mineral or carbohydrate balance as is certainly partly the case in adrenalectomy. The experimental material so far assembled permits of two possible explanations: either (1) the insufficiency symptoms in the water balance are due to medulla deficiency—the prompt effect of adrenaline, even in small dosage, strongly supports this contention; or (2) the adrenaline effect is purely secondary, merely equalizing the shock effect suffered by medullectomized animals and normal rats.

To test the above point, six medullectomized and six control rats were compared in the following experiment: A loop of small intestine was exposed through a small incision in the abdominal wall under ether narcosis and drawn by hand for 15 min. through Ringer solution. The fall in body temperature due to the shock thus inflicted averaged 4·5°C. in both groups. The recovery time in the normal animals was 2–3 hr. Two medullectomized animals recovered in the same time span, four medullectomized rats showed longer recovery times. Adrenalectomized rats died under the shock of this experiment. The difference in response between medullectomized and normal rats is slight and leaves little doubt that the effect of adrenaline is not merely one counterbalancing shock. This conclusion is further supported by the finding that neither ephedrine nor caffeine and coramine influenced the reaction. It seems probable that the effect of adrenaline is primary and is to be interpreted as a specific hormonal action. There is still, however, a further possible explanation. Although in every examined case satisfactory cortical regeneration was found, regeneration of the zona reticularis of the adrenals was in no case recorded. The possibility is present, therefore, that this and the directly adjacent tissue contain a specific substance regulating water balance. This substance might well be related to desoxycorticosterone acetate, it being significant in this connexion that in water diuresis large doses of the latter were of undoubted influence on the water excretion of the medullectomized rats. Decision between the two interpretations indicated is not yet possible.

SUMMARY

1. Urine excretion after a single water administration is abnormally low and prolonged in medullectomized rats which evince none of the known symptoms of cortical deficiency. After the first post-operative month, the inhibition of diuresis in the medullectomized rats is less marked and excretion values equal to the normal minimum values are recorded. The inhibition of diuresis in medullectomized rats and even that in adrenalectomized rats is abolished by administration of adrenaline. Chlorine excretion in the water diuresis tests was increased by adrenaline to a much more marked extent in medullectomized than in normal animals. Desoxycorticosterone acetate abolishes the inhibition of diuresis only in very high doses, but does not affect the chlorine excretion.

2. Medullectomized rats are far more susceptible to water intoxication than normal animals. Adrenaline diminishes and may abolish the difference in response between the two groups.

3. Medullectomized rats exhibit polydypsia and polyuria in particularly pronounced form in the second and third post-operative months, and in milder form subsequently. These disturbances were encountered in summer and spring but not in winter.

4. Possible causes of the described disturbances in water balance are discussed.

The authors are deeply grateful to Professor Franco and Dr Ungar for pathological-anatomical study of regenerated tissue specimens.

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THE NATURE OF THE HYPEROSSIFICATION OBSERVED IN THE LONG BONES OF RATS TREATED WITH EXCESSIVE DOSES OF OESTRADIOL BENZOATE

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A naturally-occurring, physiological process of marrow ossification was correlated by Kyes & Potter in 1934 with the size of the ovarian follicle in female pigeons. This was perhaps the first indication that female sex hormones had what is now known to be a dynamic, physiological, and pathological effect on bone.

Body weight, as early as 1931, however, was noted by Spencer, Gustavson & D'Amour to be depressed as a result of oestrogen treatment. The fact that body growth in general was markedly depressed was later verified by innumerable investigators [Spencer, D'Amour & Gustavson, 1932; Korenchevsky & Dennison, 1934; Zondek, 1936*a*, 1936*b*; Freudenberger & Clausen, 1937*a*, 1937*b*; Silberberg & Silberberg, 1939; Day & Follis, 1941]. Zondek [1936*b*] offered the first explanation of this phenomenon, showing conclusively that the oestrogenic hormones depressed the function of the anterior pituitary to the extent of inhibiting the production of the growth-promoting factor of this gland. Folley [1936] noted that the lactogenic factor of the pituitary was depressed under the influence of oestrogenic substance. Other investigators have observed that the production of the gonadotropic fraction is also inhibited [Leonard, Meyer & Hisaw, 1931; Meyer, Leonard, Hisaw & Martin, 1930, 1932; Moore & Price, 1932; Selye & Friedman, 1940; Zondek, 1936*b*].

As the body growth is depressed due to oestrogen treatment, so also is the weight and size of the experimental animal's femur. Zondek [1936*b*] noted a shortening of 19% and a drop in weight of 40% in femurs of long-treated cocks. Wentworth, Smith & Gardner [1940] working with mice, however, have observed an increased fresh weight in the femurs of mice given high doses of oestradiol benzoate. The reaction to oestrogens noted in mice, as will be mentioned later, is in direct contradiction to the picture in the rat and other experimental animals. That this might be due to species differences is suggested, but the differences in technique employed in assembling the various data have not as yet been ruled out.

In addition to this marked effect on growth of bone, it has been demonstrated by various workers that disturbance in the ossification mechanism due to the administration of oestrogens also occurs. This association was noted first by Kyes & Potter [1934] in the pigeon and later by Kirschbaum, Pfeiffer, Van Heuverswyn & Gardner [1939] in the female English sparrow in relation to the natural seasonal variation of the activity of the ovaries. Gardner & Pfeiffer [1938] produced marked evidence of increased bone formation in the marrow cavities of male mice treated with high doses of oestradiol benzoate. Similar osseous changes on administration of this

hormone were noted by Pfeiffer & Gardner [1938] in the pigeon, by Silberberg & Silberberg [1939] in the guinea-pig, and by Zondek [1936*b*] in the femurs and tibias of his oestrogen-treated chickens and rats. Sutro [1940] further verified histologically that increased trabeculation, and marked production of new endosteal bone in association with these trabeculae, took place as a result of oestrogen treatment.

In a chemical study of this problem, Wentworth *et al.* [1940] further substantiated their morphological findings in mice. They showed increased fresh weights, ash weights, and calcium and phosphorus contents of the femurs of their experimental animals. They also demonstrated that the percentage ash as well as the calcium-phosphorus ratios were increased. On the other hand, Day & Follis [1941] have found that the picture in oestrogen-treated rats differs considerably from that in the mouse. Although they agree as to the increased percentage of the ash in their animals, they note that following 104 days of treatment, the total ash, rather than increase, showed a significant decrease. In a similar chemical study in rats, we were able to substantiate the findings of Day & Follis. It was shown that following prolonged treatment with oestradiol benzoate, the femurs of the treated animals contained either the same or a slightly decreased amount of total ash (Fig. 3).

The serum balance of the elements important in the mechanism of bone formation is also disrupted by the administration of female sex hormone. Riddle & Reinhart [1926] noted a marked rise in serum calcium of female pigeons associated with ovulation. Riddle & Dotti [1936] produced significant increases in blood calcium of pigeons with gonadotropic hormone. The effect, they noted, could not be elicited in castrated animals, indicating that the action was apparently mediated through sex-hormone-producing tissues. Avery, Scott & Conrad [1940] doubled the serum calcium of immature pullets in 19 days with large doses of oestrone. Day & Follis [1941] observed a marked rise in blood calcium in the oestrogenized rat. Most recently, Landauer, Pfeiffer, Gardner & Shaw [1941] have observed that blood calcium, inorganic phosphorus and the lipids rise significantly with oestrogen stimulation. Calcaemia and lipaemia were also noted by Landauer, Pfeiffer, Gardner & Man [1939] in immature chickens treated with female sex hormones.

Although a direct relationship has been shown to exist between oestrogen treatment and a rise in serum calcium, that this is directly responsible for the observed hyperossification reported has not as yet been demonstrated.

In general, it has been observed that the animal after long oestrogen treatment is more or less dwarfed in size. It has smaller bones which present histological and chemical indications of so-called hyperossification. The bones themselves have been shown to contain more ash in mice and the same or less ash in rats when compared to controls of the same age. Similarly, the percentage ash of the de-fatted dry bone has been observed to be significantly increased. The blood picture quite conclusively shows hypercalcaemia in several species. There is also some indication of hyperphosphoraemia and hyperlipaemia.

STATEMENT OF PROBLEM

It is now definitely established that high long-continued doses of female sex hormones effect a distinct histological change in bone. Yet chemical data showing similar changes in the composition of these bones have not been as clear cut as the histo-

logical sections might indicate would be the case. The small number of investigations, complicated by the use of different species and dosage levels, tend, certainly, to confuse the picture.

The object of the present investigation has been to overcome this hiatus by an examination not only of the inorganic elements but of factors which have not so far received due attention, such as water and organic content. The data herein presented supplement the chemical picture and have given rise to a new interpretation with regard to the effect of high long-continued doses of the oestrogens on the long bones of the rat. Substantiation of previous histological observations is also offered. Correlation between the histological and chemical pictures has been considered.

METHOD

General outline

Forty-eight immature female rats were used for this study. Of these, twenty-four animals were ovariectomized and the other twenty-four left as controls. Within each of these two main groups, twelve animals were treated daily with 125 i.u. of α-oestradiol monobenzoate* dissolved in 0·1 ml. of sesame oil; the other half, litter-mates of the former twelve in each case, serving as the controls for the treated animals, received a like amount of sesame oil daily. The animals were kept in clean, dry, metal cages and fed an adequate diet.

At intervals of 1, 2, 4 and 6 weeks, three animals from each of the four groups were autopsied.

Specific procedures

(1) Ovariectomy was performed under ether anaesthesia at 22 days of age using an abdominal approach. Aseptic conditions were maintained as far as possible. The animals recovered almost completely within 2–3 hr. of the operation. The incision was allowed to seal overnight before further procedures were attempted.

(2) On the following or 23rd day, 1·0 ml. of a hypotonic solution of potassium mono-acid phosphate containing approximately 15 μcurie of radioactive potency† per ml. was injected intraperitoneally.

(3) Administration of the oestradiol benzoate was begun on the 23rd day, the experimental animals receiving 125 i.u. in 0·1 ml. of sesame oil and the controls a like amount of oil at the same time. This daily treatment was continued throughout the experiment.

The hormone was administered subcutaneously at the nape of the neck and in such a manner that the least amount of back-seepage and loss would ensue. This was accomplished by constricting the site of injection as the needle was withdrawn and massaging the oil down the back. The daily massage was used also for the purpose of aiding absorption.

(4) Body weights and vaginal smears were taken routinely every other day throughout the length of the experiment.

(5) As indicated above, twelve animals, three representatives from each group, were autopsied on the first, second, fourth and sixth weeks after the beginning of treatment.

* Received through the courtesy of the Schering Corporation, Bloomfield, New Jersey.

† Based on standard set by the Crocker Radiation Laboratory, University of California, Berkeley.

The animals were anaesthetized with chloroform and bled from the heart. The right femur was taken for chemical and radioactive analyses. The ovaries from the first two groups and right tibias from all the animals were fixed in Bouin's fluid for histological study.

RESULTS

Growth

As in previous descriptions of the effect of oestrogens on growth, oestradiol benzoate, employed in this study, caused a marked depression of body-weight increase.

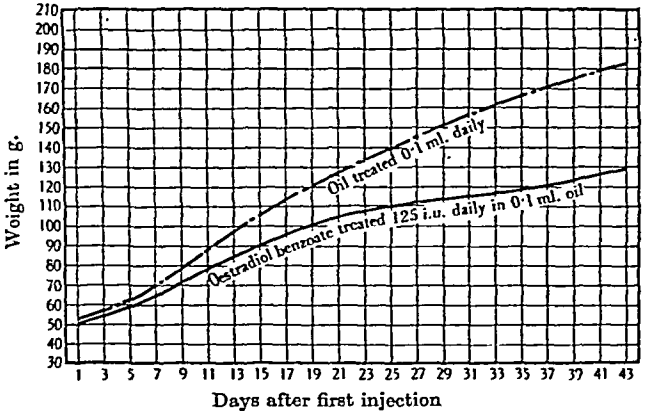


FIG. 1. Growth curves of unoperated group demonstrating the effect of oestradiol benzoate on body weight.

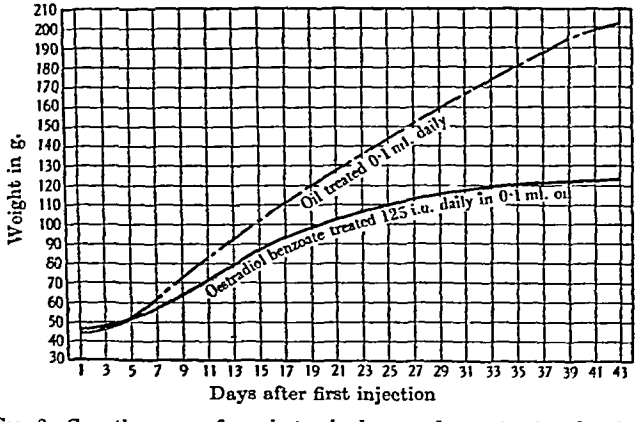


FIG. 2. Growth curves of ovariectomized group demonstrating the effect of oestradiol benzoate on body weight.

With reference to the growth curves (Figs. 1, 2), each point represents an average derived from the body weights of three animals. From these curves, the following specific observations are drawn.

During the first week, no definite effect is noted, although by the second week, the oestrogen-treated animals begin to show a tendency to decrease their rate of gain

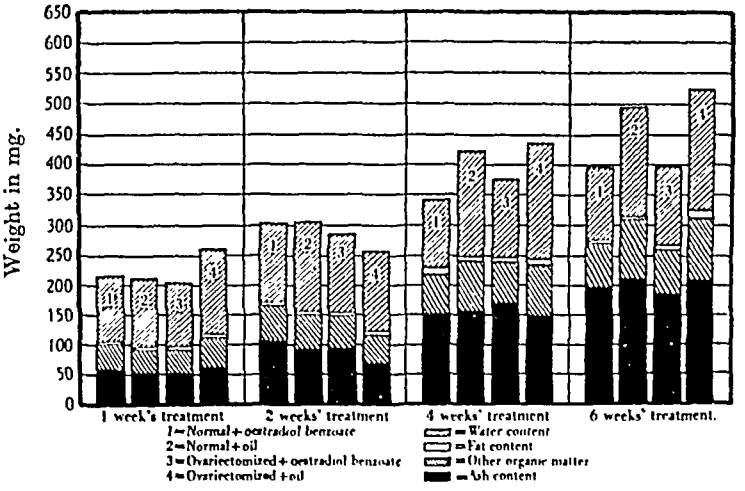


FIG. 3. Effect of oestradiol benzoate on the constituents of the right femurs of normal and ovariectomized female rats.

Table 1. *Intact rats*

Animal no.	Age at autopsy days	Oestradiol benzoate dose i.u.	Body wt. g.	Wt. of fresh femur mg.	Water mg.	Fat mg.	Rest of organic material mg.	Ash mg.
B 2211	30	875	61	200	103	4	41	52
B 2214	30	875	61	219	115	4	44	56
W 2220	30	875	66	226	115	3	46	62
		Average:	63	215	111	4	44	57
GH 2212	30	Oil	69	205	114	2	42	47
B 2215	30	Oil	69	213	113	5	43	52
W 2221	30	Oil	64	212	113	4	42	53
		Average:	67	210	113	4	42	51
B 2225	37	1750	101	326	151	4	66	105
W 2230	37	1750	95	284	116	4	57	108
W 2232	37	1750	95	300	133	5	60	103
		Average:	97	303	133	4	61	105
B 2226	37	Oil	97	277	138	4	54	82
BH 2231	37	Oil	110	311	150	6	61	94
W 2233	37	Oil	104	330	164	4	63	99
		Average:	104	306	151	5	59	92
B 2234	51	3500	106	314	101	5	62	145
BH 2295	51	3500	123	410	134	19	76	181
W 2239	51	3500	102	301	107	8	59	127
		Average:	110	342	114	11	66	151
B 2235	51	Oil	161	443	180	7	91	166
BH 2294	51	Oil	149	425	168	9	85	154
B 2240	51	Oil	143	394	167	7	80	140
		Average:	151	421	172	8	85	157
W 2241	65	5250	133	421	126	6	80	208
W 2247	65	5250	115	337	103	3	67	164
W 2252	65	5250	138	427	124	5	81	216
		Average:	129	395	118	5	76	196
W 2242	65	Oil	218	533	193	6	109	223
W 2248	65	Oil	145	411	154	4	83	169
W 2254	65	Oil	185	538	188	5	107	237
		Average:	183	494	178	5	100	210

Table 2. *Ovariectomized rats*

Animal no.	Age at autopsy days	Oestradiol benzoate dose i.u.	Body wt. g.	Wt. of fresh femur mg.	Water mg.	Fat mg.	Rest of organic material mg.	Ash mg.
B 2216	30	875	51	182	93	9	36	43
BH 2254	30	875	68	230	117	2	49	63
BH 2257	30	875	54	196	107	6	41	43
		Average:	58	203	106	6	42	50
G 2217	30	Oil	66	222	122	4	45	51
BH 2255	30	Oil	74	258	146	4	52	57
BH 2258	30	Oil	89	300	162	9	60	70
		Average:	76	260	143	6	52	59
G 2261	37	1750	80	239	108	6	47	78
B 2262	37	1750	94	293	133	5	57	98
B 2264	37	1750	106	325	156	4	63	103
		Average:	93	286	132	5	56	93
W 2260	37	Oil	81	218	114	3	43	58
B 2263	37	Oil	93	283	147	6	56	74
B 2265	37	Oil	88	269	144	4	53	68
		Average:	87	257	135	4	51	67
B 2267	51	3500	117	361	127	7	68	159
B 2269	51	3500	133	381	127	7	71	177
W 2271	51	3500	121	384	130	11	75	169
		Average:	124	375	128	8	71	168
B 2268	51	Oil	160	440	194	8	89	149
BH 2270	51	Oil	160	415	187	7	83	139
B 2272	51	Oil	158	444	190	9	89	157
		Average:	159	433	190	8	87	148
B 2273	65	5250	138	432	142	6	83	200
BH 2283	65	5250	122	354	116	10	67	161
W 2287	65	5250	118	402	121	8	74	198
		Average:	126	396	126	8	75	186
B 2274	65	Oil	213	593	236	10	118	228
BH 2284	65	Oil	174	439	160	13	87	178
W 2288	65	Oil	219	535	196	16	106	216
		Average:	202	522	197	13	104	207

in body weight. By the third week, both the unoperated and ovariectomized animals treated with oestradiol benzoate weigh about 20 % less than the oil-treated controls. Progressively, in the fourth week, the effect is increased to about 26 %, and by the fifth week to 30 %. At the end of 6 weeks of treatment, however, the treated castrated group weigh 39 % and the treated unoperated group 30 % less than the controls. This difference is due to the more rapid growth of the untreated castrated controls rather than to the more marked depression of growth in the treated castrated animals.

Chemical findings

First, it may be noted that coincident with the depression of body weight, described above, the femurs of the oestrogen-treated animals showed a marked decrease in size. By the fourth week of treatment, both the normal and ovariectomized groups showed marked and directional differences in the fresh weights of their femurs from those of their controls (Fig. 3 and Tables 1, 2). In the former, the

treated animals' femurs were about 19 % lighter and in the latter about 13 % lighter than their untreated litter-mates. At the sixth week, the differences were even more marked, the unoperated treated animals' bones being about 20 % and the castrated treated animals' femurs about 24 % lighter than their respective controls.

These differences in fresh femur weights might have been expected, considering the depression in both the size of the bone and the body weights. Less obvious, however, is the fact that these decreasing fresh weights are due almost solely to the non-asheous content of the bone, i.e. essentially the water and organic material.

Water, it was found, showed a marked decrease in both oestrogen-treated groups. It may be observed from Figs. 4 and 5 that as early as the first week, there is a decrease in the percentage water of the fresh weight and that this decrease is progressive up to the fourth week. Thereafter, up to the sixth week, the ratio of water

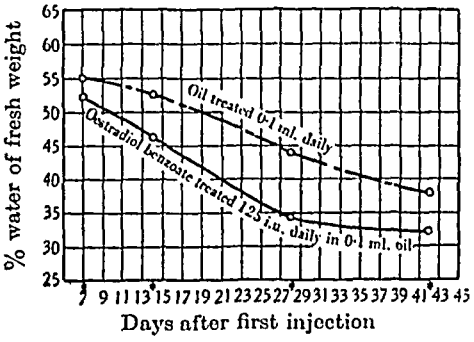


FIG. 4.

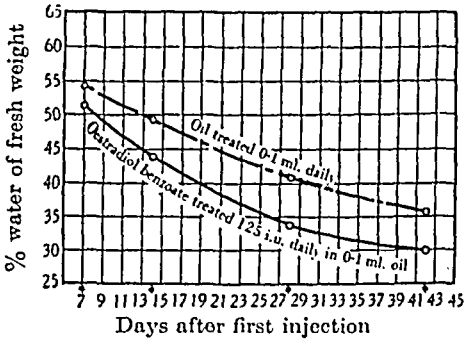


FIG. 5.

FIG. 4. Curves showing the effect of oestradiol benzoate on the percentage of water in the fresh weights of femurs of ovariectomized rats.

FIG. 5. Curves showing the effect of oestradiol benzoate on the percentage of water in the fresh weights of femurs of unoperated female rats.

‡ = Autopsy dates.

to fresh weight is maintained and shows no further appreciable decline. However, the appearance of constancy in these ratios at the sixth week is fallacious, for at this time the non-asheous substances, particularly the organic content of the bones of the treated animals, is likewise undergoing a marked decrease when compared with the controls. The decrease in organic content, therefore, tends to obscure the fact that in reality the decrease in water percentage is still progressive in the sixth week.

While the fat content varies insignificantly throughout the experiment, the absolute weights of the remaining organic material, as mentioned above, as well as its percentage of the fat-free dry bone, show a steady decrease from the fourth week in the oestradiol benzoate-treated as compared to the oil-treated animals.

Since the organic material and ash are the only two constituents of the fat-free dry bone, it is obvious that as the percentage of the organic material gradually decreases, the percentage ash of the fat-free dry weight would reciprocally increase (Figs. 6, 7). As a matter of fact, it can be seen from Fig. 3 and Tables 1 and 2 that the absolute amount of ash actually decreases rather than increases in the oestrogenized animals at the sixth week of treatment.



Fig. 8.



Fig. 9.

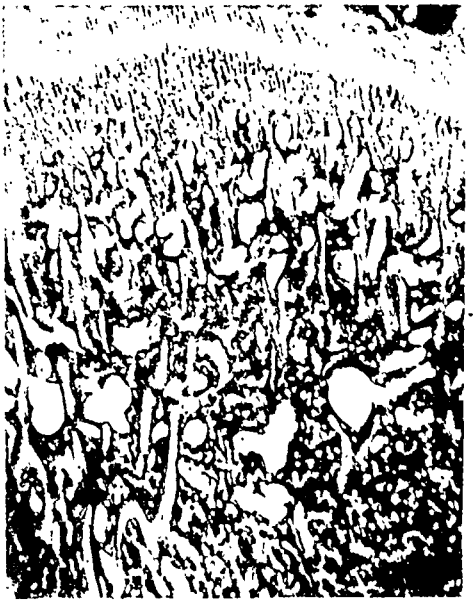


Fig. 10.



Fig. 11.

Plate 1. The effect of chronic excessive doses of oestradiol benzoate on the number, length, and thickness of the bony trabeculae and the amount of endosteal bone formation in the tibias of immature female rats. Fig. 8. Unoperated control treated for 6 weeks with oil only. Fig. 9. Normal animal after 6 weeks of daily treatment with 125 i.u. of oestradiol benzoate. Fig. 10. 6-week ovariectomized oil-treated control. Fig. 11. Ovariectomized animal after 6 weeks of daily treatment with 125 i.u. of oestradiol benzoate. Magnification $\times 32{\cdot}5$.

It is observed that neither the absolute calcium and phosphorus weights nor their percentages of the ash show any significant directional variation in the treated animals when compared to their controls.

The calcium and phosphorus values increase in both treated and control groups with the age of the animals. The ratio of calcium to ash in both groups shows, perhaps, a slight decrease with age, while the ratio of phosphorus to ash varies insignificantly within a small range from the first to the sixth week.

The calcium-phosphorus ratio shows no significant change in either treated or untreated animals, but decreases slightly with age.

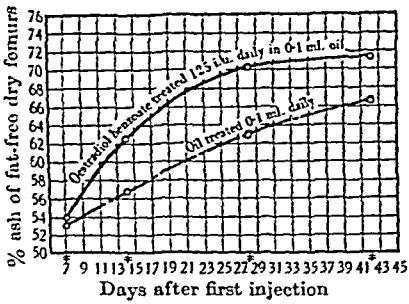


FIG. 6.

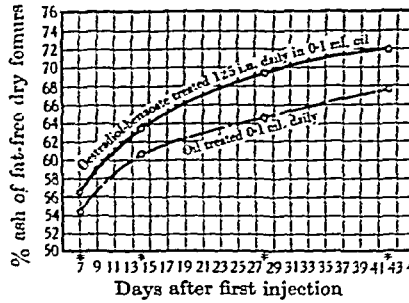


FIG. 7.

FIG. 6. Curves showing the effect of oestradiol benzoate on the percentage ash of the fat-free dry femurs of ovariectomized rats.

FIG. 7. Curves showing the effect of oestradiol benzoate on the percentage ash of the fat-free dry femurs of normal unoperated female rats.

‡ = Autopsy dates.

While definite conclusions concerning the effect of oestradiol benzoate on the rate of metabolism of radioactive phosphorus are questionable from this study, there are certain tendencies to be noted which require further study utilizing a larger group of animals. It is observed that in the majority of instances, the amount of radioactive phosphorus present was slightly greater in oestrogen-treated animals than in their controls. This statement must be further modified for the second week group, in which, unexplainable by other data, both the untreated normals and especially the castrated animals showed a marked, sudden increase in metabolic activity over the experimental animals. While the variability and overlapping of data tend to minimize the significance of these findings, the impression is gained, nevertheless, that oestradiol benzoate treatment causes a slight increase in the metabolic rate of radioactive phosphorus.

Histological and miscellaneous observations

Plate 1, figs. 8–11, demonstrate quite definitely the so-called hyperossification effect of oestrogens on the long bones of rats.

In both groups treated with oestrogen, marked trabeculation is apparent by the fourth week. There is no significant increase during the first week, although in the second week the tendency to increased ossification is noted. By the fourth and sixth weeks there is a complete new organization of bony trabeculae with marked increase in number, fusion, and thickening of the normally thin, sparse, bony

cords. In many cases, the treated animals showed trabeculae extending much farther down into the marrow cavity than in the untreated controls, thereby decreasing to some degree the medullary canal. The area of provisional ossification was a thick bony mass by the sixth week. The ossification appeared to be wholly endosteal in origin.

The epiphyseal disks of both groups of oestrogen-treated animals showed a comparable progressive decrease in thickness from the first to the sixth week of treatment, the effect diagrammatically describing a logarithmic curve. When compared with their respective controls, however, although the disks of the experimental animals were always narrower than those of their controls, these differences were less marked in both groups by the sixth week.

Ovarian weights showed a progressive depression due to treatment with oestradiol benzoate. Both grossly and histologically the ovaries appeared immature in oestrogen-treated animals when compared with their litter-mate controls.

In all treated cases, the oestradiol benzoate maintained the animals in a constant state of vaginal cornification, as indicated by smears. This condition was modified by secondary effects on the uterus and vagina. For the first week or two, the smears were definitely cornified indicating the stage of oestrus; but as the effect was prolonged, the smear became thick with mucus and pus and was greenish in colour showing, in general, varying numbers of leucocytes and small epithelial cells scattered throughout the thick cornified cells. This state continued for the duration of the experiment. There was no apparent difference between the castrated and intact treated groups with regard to the vaginal smear. The smears of the normal oil-treated controls showed regular cycles following the opening of the vaginal orifice. The castrated, oil-treated controls showed smears in all stages of L. and E. (leucocytes and epithelial cells), varying from a few leucocytes and desquamated epithelial cells to many leucocytes and no epithelial cells.

The vaginal orifices of the treated animals opened 4–6 days after the start of treatment; those of the control animals opened quite variably. The normal group opened in 11–15 days with one exception and that one animal easily admitted a spatula at a later date indicating that the vagina was merely blocked by disintegrated strands of tissue. The castrated controls opened in 9–13 days, with two exceptions which remained closed for the duration of the experiment, i.e. until they were killed at 4 weeks.

DISCUSSION

The data and results herein presented have in most instances substantiated the findings of the majority of investigations made on this problem. Oestrogens have been observed to cause a marked depression of body-weight increase. The increase consistently observed in the percentage ash of the bones confirms the work of Wentworth *et al.* [1940] in the mouse, and Day & Follis [1941] in the rat. The decreased ash content of the femurs of oestrogenized rats observed in the later periods of treatment is similar in degree to that found by Day & Follis in the rat. Histologically, the progressive picture of increased trabeculation and endosteal bone growth is noted. Yet, it is felt that this is not the whole picture. As will be discussed, additional data on the decrease in water and organic contents of the bones, due to oestrogen stimulation, show these factors to play a much more important role than has been suspected

These factors, in our opinion, offer evidence of the relative nature of the hyperossification.

The Silberbergs [1939] have rightly observed that the effect of oestrogens on skeletal tissue '...cannot be defined as mere ossification of the epiphyseal disks with subsequent cessation of growth in lengthwise direction...nor as hyperostotic osteo-sclerotic growth in the shaft and marrow cavity of the long bones'.

The effect of oestrogens on bone is not simply a stimulation to new ossification as is evidenced by the above observations. If, as previous workers have suggested, the process were one of hyperossification *per se*, it would be expected that the bones would contain an increased amount of ash.

Hammett [1925] first suggested the relationship between female sex hormones and water metabolism of bone. He observed that female rats reaching physiological puberal development showed a significant decrease in the water content of their bones. That this was associated with ovarian stimulation by the gonadotropic hormones of the pituitary was not noted, although such an interpretation is certainly not unreasonable. The differential distribution of water due to oestrogen stimulation was observed by Zuckerman, Palmer & Bourne [1939]. They noted that skin, uterus and vagina showed a primary increase followed by a decrease and a secondary and more stationary increase in water content. Striated muscle, heart, pancreas, brain, and gut, on the other hand, displayed a reciprocally opposite reaction. Although bone was not studied, the evidence herein offered would indicate that it belonged to the latter of the two groups of differentially responding tissues. Our investigations reveal that with chronic oestrogen stimulation, there is a marked and progressive decrease in water content of the long bones of rats. In addition, the organic content of these bones was also noted to be significantly decreased. Wentworth *et al.* [1940] reported a decreased organic content in the bones of their treated mice, but did not associate this with similar deviations in water content. The decreased organic and water content of the femur of the treated rat, however, are the factors which make the hyperossification described in that animal apparent. Also, in contradiction to Wentworth *et al.* [1940], we were not able to show any significant increase in the calcium and phosphorus content and the calcium-phosphorus ratios of the bones of treated rats.

Therefore, in analysing the basis for the observed phenomenon of hyperossification in the rat, we are left with the one fact that the percentage ash in the bones of oestrogen-treated rats is constantly increased. This may be due either to an increase in the ash content or a decrease in the non-asheous content of these bones. We have shown, however, that the ash content remains essentially unchanged in both treated and untreated groups, and further that the non-asheous content is decreased markedly in the treated animals. It will be remembered that the growth in terms of body weight is markedly depressed in the oestrogen-treated rat. If then we correct the values of the non-asheous content to the body weight in order to eliminate this as a factor, it is noted that the relationship of the non-asheous to the asheous content is essentially the same in the bones of both treated and untreated groups (Tables 3, 4). Graphic representation of this fact is seen in Figs. 12 and 13, where it is noted that at the time when the greatest amount of osseous concentration is taking place—after 4 and 6 weeks—the ratios for the controls and the ratios for the treated animals

corrected to body weight practically coincide in both the operated and unoperated groups. The fact that a concentration of ash takes place is not disputed, but an attempt is being made to obviate those factors which make it apparent. These facts suggest the conclusion that the effect of oestrogens is not one of direct stimulation to bone growth or hyperossification but rather a decreased stimulation of body growth in general. This has been shown previously to be due to the depressing effect of the oestrogenic hormone on the anterior pituitary body. The depression of body growth is manifested in the bone as a negative water and nitrogen balance. It is possible that the more labile constituents of the bone will respond more readily to this process than the osseous portion. Perhaps a more valid interpretation would be that the two processes—body growth and bone ossification—enjoy separate control, the former in this case being disrupted through the pituitary while the latter continues without restriction. It is not difficult to imagine that such a process would result in the smaller, more compact bone seen in the animals treated with high dose of oestradiol benzoate.

Table 3. *Intact rats*

Animal no.	Age at autopsy days	Oestradiol benzoate dose i.u.	Non-ash content mg.	Ratio non-ash/ash: body wt. ×1000
B 2211	30	875	148	46·72
B 2214	30	875	163	47·38
W 2220	30	875	164	39·91
		Average:	158	44·67
GH 2212	30	Oil	158	49·18
B 2215	30	Oil	161	44·82
W 2221	30	Oil	159	46·65
		Average:	159	46·88
B 2225	37	1750	221	20·75
W 2230	37	1750	177	17·34
W 2232	37	1750	197	20·17
		Average:	198	19·42
B 2226	37	Oil	195	24·63
BH 2231	37	Oil	217	20·90
W 2233	37	Oil	231	22·39
		Average:	214	22·64
B 2234	51	3500	169	10·93
BH 2295	51	3500	229	10·31
W 2239	51	3500	174	13·49
		Average:	191	11·58
B 2235	51	Oil	277	10·38
BH 2294	51	Oil	261	10·68
B 2240	51	Oil	255	12·77
		Average:	264	11·28
W 2241	65	5250	213	7·697
W 2247	65	5250	173	9·149
W 2252	65	5250	212	7·112
		Average:	199	7·986
W 2242	65	Oil	310	6·369
W 2248	65	Oil	242	9·882
W 2254	65	Oil	301	6·849
		Average:	284	7·700

Table 4. *Ovariectomized rats*

Animal no.	Age at autopsy days	Oestradiol benzoate dose i.u.	Non-ash content mg.	Ratio non-ash/ash : body wt. × 1000
B 2216	30	875	139	62·63
BH 2254	30	875	168	39·44
BH 2257	30	875	153	65·79
		Average:	153	55·95
G 2217	30	Oil	171	50·64
GH 2255	30	Oil	202	48·22
BH 2258	30	Oil	231	37·05
		Average:	201	45·30
G 2261	37	1750	161	25·87
B 2262	37	1750	195	21·10
B 2264	37	1750	222	20·42
		Average:	193	22·46
W 2260	37	Oil	160	33·98
B 2263	37	Oil	209	30·49
B 2265	37	Oil	201	33·86
		Average:	190	32·78
B 2268	51	3500	202	10·89
B 2269	51	3500	205	8·726
W 2271	51	3500	216	10·55
		Average:	208	10·06
B 2268	51	Oil	291	12·21
BH 2270	51	Oil	277	12·49
B 2272	51	Oil	287	11·62
		Average:	285	12·11
B 2273	65	5250	232	8·412
BH 2283	65	5250	193	9·802
W 2287	65	5250	213	9·142
		Average:	213	9·119
B 2274	65	Oil	365	7·521
BH 2284	65	Oil	261	8·419
W 2288	65	Oil	320	6·763
		Average:	315	7·568

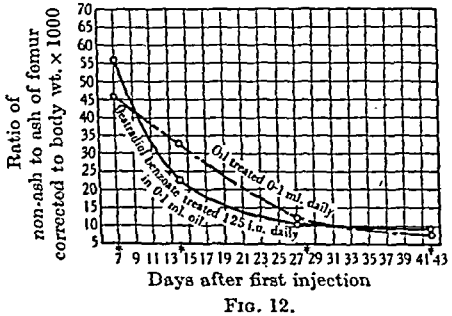


Fig. 12.

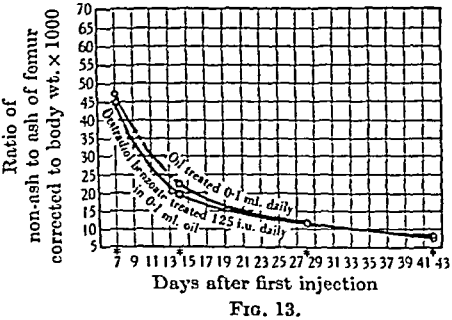


Fig. 13.

Fig. 12. Curves showing the effect of oestradiol benzoate on the ratio of non-ash to ash contents of the femurs corrected to body weight in ovariectomized rats.

Fig. 13. Curves showing the effect of oestradiol benzoate on the ratio of non-ash to ash contents of the femurs corrected to body weight in normal female rats.

‡ = Autopsy dates.

It is our contention, then, that the effect of high doses of oestrogenic substances on bone is part of the general effect the hormone has been shown to have on body growth, namely, depression. In the case of bone, secondary factors produce what we would term a secondary or relative hyperossification which when examined microscopically appears as an increased number and thickness of trabeculae; but when examined chemically appears merely as a relative reduction in two of the three main constituents of bone—namely, water and organic material.

CONCLUSIONS

The following conclusions are drawn relating to the effects of daily subcutaneous injections of 125 i.u. of oestradiol benzoate in sesame oil over a period of 1–6 weeks into immature, unoperated and ovariectomized, female rats.

1. Oestradiol benzoate has been shown to cause a significant progressive decrease in the water content of the femurs of both unoperated and ovariectomized rats.

2. The absolute organic content of the femurs of the oestrogenized animals has been observed to be markedly decreased by the fourth and progressively further decreased by the sixth week of treatment.

3. The ash contents of the femurs of oestrogen-treated rats were observed to vary insignificantly until the sixth week at which time they were significantly less than those of their oil-treated controls.

4. Body-weight increase was markedly and progressively depressed as a result of treatment with oestradiol benzoate in both normal and castrated rats.

5. The percentage ash of the fat-free dry femurs of oestrogenized rats showed progressive and significant increase over those of oil-treated controls.

6. Markedly increased trabeculation and the formation of apparently new endosteal bone by the fourth week of treatment with oestradiol benzoate was observed histologically in the tibias of both normal and ovariectomized rats.

7. The percentage calcium and percentage phosphorus of the femur ash showed no significant difference between treated and untreated rats.

8. The calcium-phosphorus ratios of the femurs of oestrogen-stimulated rats varied insignificantly as compared to their oil-treated controls.

9. Evidence has been presented and a discussion offered in interpretation of the histological and chemical picture seen in the long bones of the oestrogen-treated rat; an interpretation which is concluded to be presumptive evidence of the relative rather than absolute nature of the previously observed so-called hyperossification.

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THE PRODUCTION OF OVULATION IN THE IMMATURE RAT

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Rowlands & Williams [1943] recently reported that the consecutive injection of mare-serum gonadotrophin and chorionic gonadotrophin would cause ovulation in hypophysectomized rats. Serum gonadotrophin was given to stimulate the growth of the atrophic follicles and, subsequently, chorionic and other gonadotrophins containing an excess of the luteinizing hormone were used as a means of causing their rupture. Ova were found in the Fallopian tubes 1–2 hr. after their discharge from the ovaries. Similar experiments have been carried out in the intact immature rat to study, in greater quantitative detail, the optimal conditions necessary for ovulation. A comparative study of the hormonal control of ovulation in different species seemed desirable, particularly on account of the disappointing results that have been frequently reported on the use of mare-serum gonadotrophin in causing ovulation in women.

MATERIAL AND METHODS

Gonadotrophic substances

Two preparations of mare-serum gonadotrophin were used: (1) the International Standard containing 4 International Units (i.u.) per mg., and (2) PMS 18, a commercial preparation containing 65–70 i.u./mg. One commercial preparation of chorionic gonadotrophin (UP 31) containing 200 i.u./mg. was used.

Administration of gonadotrophins

Each substance was dissolved in water and administered as a single subcutaneous injection. At least ten immature female rats weighing 40–50 g. were used for each experiment.

Criterion of ovulation

The occurrence of ovulation was detected by direct observation of the eggs in the Fallopian tubes when in the fresh condition, as described by Rowlands [1942]. After dissection of the Fallopian tube under a binocular dissecting microscope the egg-containing segment was excised and the eggs, each surrounded by a dense mass of cumulus cells, were expressed and counted. The ovaries were fixed in Bouin's fluid overnight and were weighed from 70 % alcohol on a torsion balance.

RESULTS

The ovulation-producing capacity of mare-serum gonadotrophin

The ovary of the immature rat weighing 40–50 g. is extremely sensitive to stimulation by various gonadotrophic substances, but its maximum quantitative response is greater to mare serum than to any other gonadotrophin [Emmens, 1940]. The

capacity of this gonadotrophin to produce ovulation in the immature rat, however, is small and is exceeded by that of extracts of horse and gelding pituitary glands [Rowlands & Williams, 1941]. In other species, also, notably the monkey [Hartman, 1938] and man [Geist, Gaines & Salmon, 1941; Brewer, Jones & Skiles, 1942], mare-serum extracts are relatively ineffective in producing ovulation. In the hypophysectomized rat, according to our experience, this gonadotrophin is quite incapable of producing ovulation when given as a single injection.

Table 1 shows that ovulation did not invariably occur in immature rats treated with amounts of mare-serum gonadotrophin varying between 2 and 60 i.u. These results are in striking contrast to those of Cartland & Nelson [1938], who obtained ovulation in over 90% of a group of twenty-five rats given three injections of the serum extract, the rats being killed 96 hr. after the first injection.

Table 1. *The production of ovulation in the intact immature rat with a single injection of International Standard mare-serum gonadotrophin. Each group consists of ten rats*

Amount of gonadotrophin injected i.u.	Time when killed after injection hr.	Weight of ovaries mg.	Ovulation %	No. of ova per rat ovulating
2	74	11	0	0
5	74	21	50	5
10	42	16	0	0
10	66	22	0	0
10	74	40	0	0
10	90	21	30	5
10	114	19	40	4
10	162	18	20	1·5
20	74	29	0	0
20	96	65	0	0
30	70	75	10	?
30	74	70	10	4
30	78	85	60	?
30	84	66	30	?
30	90	94	30	?
30	96	93	0	0
30*	90	64	10	1
60	74	40	10	1

* Three injections of 10 i.u. once daily.

The experiments recorded below were designed to test whether the injection of chorionic gonadotrophin (luteinizing hormone) would readily cause ovulation in an ovary previously stimulated with mare-serum gonadotrophin (follicle-stimulating hormone). Similar experiments using the follicle-stimulating hormone and luteinizing hormone from pituitary gland have already been carried out with success on the anoestrous cat by Foster & Hisaw [1935], on the hypophysectomized rabbit by Foster, Foster & Hisaw [1937], and on the anoestrous opossum by Nelsen & White [1941]. The latter authors conclude that a 'proper stimulation of follicular development with relatively pure F.S.H. should precede injections containing the luteinizing factor in order consistently to bring about ovulation in the anoestrous opossum....'

Ovulation produced by mare-serum gonadotrophin and chorionic gonadotrophin

Varying amounts of mare-serum gonadotrophin and a constant amount of chorionic gonadotrophin. Groups of ten rats were injected with varying amounts of mare-serum gonadotrophin. From previous experience it had been ascertained that ovulation occurs about 70–80 hr. after this treatment, and our recent results on hypophysectomized rats [Rowlands & Williams, 1943] showed that a maximum period of 15–18 hr. is necessary for ovulation to occur following the subcutaneous injection of chorionic gonadotrophin. It was assumed that the same conditions would hold good for intact rats, so that 56 hr. after the injection of mare-serum gonadotrophin a subcutaneous injection of 20 i.u. of chorionic gonadotrophin was given and 18 hr. later the Fallopian tubes were examined for the presence of ova. The over-all period of the test was, therefore, 74 hr. The results obtained are given in Table 2.

Table 2. *The effect of a constant amount of chorionic gonadotrophin* (20 *i.u.*) *on rats injected* 56 *hr. previously with varying amounts of mare-serum gonadotrophin. Each group consisted of ten rats and all were killed* 18 *hr. after the injection of chorionic gonadotrophin*

Amount of serum gonadotrophin i.u.	Weight of ovaries mg.	Ovulation %	No. of ova per rat ovulating
0	10	0	0
0·25	18	10	7
0·5	16	0	0
0·75	20	40	3
1·0	18	80	4
1·5	18	100	4
2·0	19	100	4·5
2·0*	11	0	0
5·0	22	100	5
5·0*	21	50	5
10·0	33	90	11
10·0*	27	0	0
20·0	46	90	14
20·0*	29	0	0
30·0	94	100	26
30·0*	70	10	4
40·0	45	60	9
50·0	41	40	5
60·0	55	30	3
60·0*	49	10	1

* Control group injected with mare-serum gonadotrophin only.

It can be seen that the injection of as little as 1·5 i.u. of mare-serum gonadotrophin followed 56 hr. later by 20 i.u. of chorionic gonadotrophin caused ovulation in 100% of animals, whereas 2 i.u. of the serum gonadotrophin without the stimulation provided by the chorionic gonadotrophin were completely ineffective. The number of ova counted in the Fallopian tubes, however, remained constant at about 5 ova per rat, until 5 i.u. of mare-serum gonadotrophin were injected. Higher dosages caused

the maturation of larger numbers of follicles with the result that a larger number of eggs were found in the tubes. The maximum number of ova (26) produced in the tubes of a rat by this technique resulted from the injection of 30 i.u. of mare-serum gonadotrophin and 20 i.u. of chorionic gonadotrophin.

The injection of amounts of serum gonadotrophin greater than 30 i.u. caused a progressive decrease in the percentage of animals ovulating and also in the number of ova discharged into the Fallopian tubes. It is probable that this was caused by over-stimulation of the follicles with the formation of follicular cysts, or by the action of amounts of the luteinizing hormone in this gonadotrophin sufficient to cause a premature luteinization of the membrana granulosa which rendered the follicles incapable of rupture.

Constant amounts of mare-serum gonadotrophin and varying amounts of chorionic gonadotrophin. Four groups each consisting of sixty immature rats were injected with amounts of mare-serum gonadotrophin ranging between 10 and 60 i.u., as shown in Table 3. After 56 hr. each group was divided into six equal subgroups, five of which were injected with varying amounts of chorionic gonadotrophin. All rats were killed 18 hr. later and the Fallopian tubes examined for the presence of eggs.

Table 3. *The effect on ovulation of varying amounts of chorionic gonadotrophin in rats injected, 56 hr. previously, with constant amounts* (10, 20, 30 *and* 60 *i.u.*) *of mare-serum gonadotrophin. Each subgroup consisted of ten rats and all were killed 18 hr. after the injection of chorionic gonadotrophin*

Amount of chorionic gonadotrophin injected i.u.	Amount of International Standard mare-serum gonadotrophin injected (i.u.) 10		20		30		60		PMS 18 (i.u.) 30	
	Ovulation %	No. of ova per rat ovulating	Ovulation %	No. of ova per rat ovulating	Ovulation %	No. of ova per rat ovulating	Ovulation %	No. of ova per rat ovulating	Ovulation %	No. of ova per rat ovulating
0	0	0	0	0	10	4	20	2	0	0
2	0	0	0	0	10	10	10	3	50	16
5	0	0	10	12	40	12	0	0	50	10
10	100	10	100	7·5	70	27	10	1	90	13
20	100	8	90	14	100	26	0	0	100	23
40	100	12	90	17	100	23	30	5	100	26

The data given in Table 3 show that although chorionic gonadotrophin contains the factor necessary for the production of ovulation, the extent of ovulation, that is, the number of ova discharged into the Fallopian tubes, is determined by the amount of serum gonadotrophin previously administered. The average number of ova found in the tubes of those rats treated with 10, 20 and 30 i.u. of serum gonadotrophin and with an amount of chorionic gonadotrophin sufficient to produce 100% ovulation was 10, 13 and 25 respectively. With one exception doubling or even quadrupling of the dosage of the latter hormone did not significantly increase in the number of eggs released from the ovary. Superovulation was caused by the administration of 30 i.u. of serum gonadotrophin and 10–40 i.u. of chorionic gonadotrophin, and a similar result was obtained by the use of the same amount of another preparation

(PMS 18) of serum gonadotrophin. The injection of 60 i.u. of mare-serum gonadotrophin stimulated the ovaries to such an extent that subsequent ovulation by chorionic gonadotrophin was almost completely inhibited.

Varying intervals between the injection of constant amounts of both serum and chorionic gonadotrophins. In all the experiments so far described 56 hr. elapsed between the administration of serum and chorionic gonadotrophins. This interval was chosen for the reason previously given (see p. 386). Although satisfactory results were obtained it seemed desirable, nevertheless, to ascertain the effect of varying this interval, particularly as this should yield some information on the time required by a known dose of mare-serum gonadotrophin to bring the Graafian follicles of the rat ovary to maturity. Experiments were therefore carried out in which 10 i.u. of mare-serum gonadotrophin were followed, at intervals of 0–144 hr., by 20 i.u. of chorionic gonadotrophin. All animals were killed 18 hr. later. The results are given in Table 4.

Table 4. *The effect on ovulation of varying the interval between a constant dose of mare-serum gonadotrophin (10 i.u.) and a constant dose of chorionic gonadotrophin (20 i.u.). Each group consisted of ten rats and all were killed 18 hr. after injection of chorionic gonadotrophin*

Interval between injections hr.	Time between injection of serum gonadotrophin and killing hr.	Weight of ovaries mg.	Ovulation %	No. of ova per rat ovulating
0	18	22	0	0
8	26	22	20	2
18	36	21	30	1·5
24	42	21	60	4
	42*	16	0	0
32	50	33	80	11
48	66	31	90	5
	66*	22	0	0
56	74	40	100	8
	74*	40	10	4
72	90	36	80	7·5
	90*	21	10	6
96	114	33	90†	3
	114*	19	50	4
144	162	22	20	5
	162*	18	20	1·5

* Mare-serum gonadotrophin only.

† In only 50% of this group was ovulation recent and attributable to the effect of injection of chorionic gonadotrophin.

Ovulation did not occur when both substances were administered simultaneously (interval = 0 hr.). The occurrence of ovulation in some of the rats injected at intervals of 8–18 hr. indicated the rapidity with which serum gonadotrophin acts on the ovaries of the immature rat. The optimal interval between the injections for the induction of ovulation was 48–72 hr.; the best result was obtained with a 56-hr. interval. All the controls of groups with injection intervals of 24, 48 and 72 hr., killed at 42, 66 and 90 hr. respectively, failed to ovulate, but in the next control

group, killed 114 hr. after injection with mare-serum gonadotrophin, 50% of the rats had ovulated. In the corresponding group injected with chorionic gonadotrophin after 96 hr. and killed at 114 hr., although ova were found in 90% of the rats, it is probable that in only 50% did ovulation occur in response to chorionic gonadotrophin. In the remaining four rats, the eggs, which were scattered along the entire length of the Fallopian tubes, were free from corona radiata and adhering cumulus cells, and several showed signs of degenerative fragmentation. It is concluded, therefore, that ovulation in these rats had occurred in response to mare-serum gonadotrophin before the injection of chorionic gonadotrophin. In one of the two rats injected with chorionic gonadotrophin 144 hr. after the serum gonadotrophin, the eggs were in the same condition and position as those described above, so that only one of the ten rats treated in this manner ovulated as the result of injection of chorionic gonadotrophin. It is obvious, therefore, that when the interval is greater than 72 hr. the capacity of the follicles, stimulated with mare-serum gonadotrophin, to ovulate in response to a subsequent injection of chorionic gonadotrophin is greatly diminished. It is probable that this is caused by degenerative changes in the mature follicles.

Time of ovulation

In hypophysectomized rats, similarly treated with mare-serum and chorionic gonadotrophins, it was found that ova began to appear in the Fallopian tube about 14 hr. after the subcutaneous injection of chorionic gonadotrophin. A comparable experiment was carried out in intact rats, the results of which are shown in Table 5.

Table 5. *Time of ovulation. All rats were injected with* 20 *i.u. mare-serum gonadotrophin and again* 56 *hr. later with* 40 *i.u. chorionic gonadotrophin* (*UP* 31)

Time when killed after injection of UP 31 hr.	Weight of ovaries mg.	No. of rats with ova in tubes %	Average no. of ova per rat ovulated
12	41	10	1
14	46	50	11
16	33	100	20
18	40	100	27

Four groups, each consisting of ten rats, were injected with 20 i.u. of mare-serum gonadotrophin, and 56 hr. later they all received an injection of 40 i.u. of chorionic gonadotrophin. The groups were killed at 2-hourly intervals 12–18 hr. afterwards. Ova were present in the tubes of all rats in the group killed at the 16th hr. In the hypophysectomized rat the eggs first appear in the Fallopian tubes about 1 hr. after their release from the ovary. Assuming that the rate of transit of the ova is the same in the intact rat, then ovulation occurred about 15 hr. after the administration of the chorionic gonadotrophin.

DISCUSSION

The experiments described above have had as their main object the determination of the optimal conditions for follicular growth and the induction of ovulation in the intact immature rat by means of the most readily available gonadotrophic hormones, viz. mare-serum gonadotrophin and chorionic gonadotrophin. The changes in the ovary necessary for the occurrence of ovulation have been brought about to a large

extent in two independent stages, i.e. follicular maturation has been induced by mare-serum gonadotrophin (follicle-stimulating hormone) and the rupture of the ripe follicles by chorionic gonadotrophin (luteinizing hormone). Efforts to produce both these changes in the rat by mare-serum gonadotrophin alone have met with little success in spite of the findings of Cartland & Nelson [1938]. In my experience, mare-serum gonadotrophin is deficient in the particular activity necessary for the occurrence of ovulation in the rat, and it is conceivable that the disappointing results obtained in man might be explained on this basis. Nevertheless, this gonadotrophin, in the correct dosage, serves admirably to bring about follicular maturation in the rat, and it was found that if chorionic gonadotrophin be injected subcutaneously 48–72 hr. later, ovulation occurs in 100% of the test animals. If a longer interval between the injections elapses, the number of animals in which ovulation occurs decreases. Follicular maturation occurs in 48–72 hr. and corresponds to the rate of follicular growth in the normal oestrous cycle of the adult rat [Long & Evans, 1922]. It may well be, therefore, that for the induction of ovulation the follicles should be stimulated at the rate which is natural for the normal adult animal of the same species. In the rat it is not known whether supra-optimal doses of serum gonadotrophin cause an accelerated rate of follicular maturation, but it was found that such doses, when followed in 2–3 days' time by chorionic gonadotrophin, had caused an over-stimulation which had prevented the release of all but a few ova.

A comparison with the results obtained by the use of hypophysectomized rats [Rowlands & Williams, 1943] shows that a greater amount of serum gonadotrophin is required to cause follicular maturation in ovaries rendered atrophic by hypophysectomy. Moreover, the time taken to cause follicular maturation in the hypophysectomized rat is nearly twice as long as it is in the intact rat. The amount of chorionic gonadotrophin required by the hypophysectomized rat to produce the maximal effect is also greater than that needed by the intact rat. This could be accounted for by the absence of any endogenous gonadotrophic (luteinizing) hormone from the circulation of the hypophysectomized rat. The intact rat probably contains a small amount of this hormone which, although not sufficient to cause ovulation by itself, augments the action of similar hormone (chorionic gonadotrophin) administered by injection. The maximum numbers of ova discharged in both test animals are very similar.

SUMMARY

1. The induction of ovulation in intact immature rats by the consecutive administration of single subcutaneous doses of mare-serum gonadotrophin and chorionic gonadotrophin is described.

2. Serum gonadotrophin, although effective in producing follicular growth, had only a limited capacity to produce ovulation.

3. Ovulation (10 ova/rat) was induced by 10 i.u. of serum gonadotrophin followed 48–72 hr. later by 10 i.u. of chorionic gonadotrophin. Superovulation was produced when the dose of the serum preparation was increased to 30 i.u., but further increase caused over-stimulation in the ovary which prevented the discharge of the ova from the follicles. Ova appeared in the Fallopian tubes 14–16 hr. after the injection of chorionic gonadotrophin.

4. The responses in intact and hypophysectomized rats are compared.

The preparation of mare-serum gonadotrophin, PMS 18, was kindly supplied by Lovens Kemiske Fabrik, Copenhagen, and the chorionic gonadotrophin, UP 31, by Organon Laboratories Ltd.

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ADDITIONAL STUDIES OF THE EXTRATHYROIDAL METABOLISM OF IODINE

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Data reported from this laboratory [Chapman, 1941] showed that thyroidectomized animals receiving large amounts of iodine lost less weight, utilized their food better, drank less water and had a higher basal oxygen consumption than those which received small amounts of iodine. The conclusion was indicated that iodine may play a role in body metabolism, in the absence of the thyroid gland.

In that initial study [Chapman, 1941], the thyroids were removed before the animals were placed on the experimental low-iodine diet. In a subsequent study, leading to this report, all animals were placed on the experimental diets for 95 days before their thyroid glands were removed.

METHODS

Twenty male rats of a Wistar strain, aged 8 weeks, were placed on a low-iodine diet as described by Remington [1937]. All animals were given distilled water to drink, but a supplement of 1·5 μg. of iodine, as potassium iodide, was added to each cubic centimetre of the drinking water of ten of them. All twenty animals were maintained on this diet for 95 days. Complete thyroidectomy was then performed on five of each group of ten and a sham operation was performed on the rest.

One hundred and twenty-six days after operation the experiment was concluded. The weights of all animals were recorded at the beginning of the experiment, at the time the operation was performed, and at the end of the experiment. By means of a closed-circuit apparatus, such as was used in the first experiment [Chapman, Baldes & Higgins, unpublished data], the basal oxygen consumption was determined at the end of the preoperative control period and six times during the postoperative period.

RESULTS

After 95 days on the low-iodine diet those animals which received the daily supplement of iodine as potassium iodide in their drinking water were not significantly heavier than those on the diet alone (Table 1). Thus, in intact animals, the growth curve was not greatly modified by the amounts of iodine consumed.

Table 1. *Effects of iodine intake on normal growth*

Diet	Animals	Average weight of animals in g. At beginning of exp.	Average weight of animals in g. After 95 days; just before thyroidectomy
Low iodine plus supplemental iodine	10	78·5 ± 1·7*	236·5 ± 5·1
Low iodine	10	78·7 ± 1·3	223·2 ± 4·6

* Probable error of the mean.

Not all animals survived the postoperative period of 126 days. Two died in the group which was fed the diet plus the supplemental iodine: one in the control group, in which a sham operation had been performed, and one in the thyroidectomized group. Likewise two died in the group which was fed the unsupplemented low-iodine diet. Both of these had been thyroidectomized (Table 2). Thus, at the

Table 2. *Effect of intake of iodine on weight of thyroidectomized and non-thyroidectomized animals*

Diet	Condition of animal	Animals	Mean weight of animals in g. At operation	Mean weight of animals in g. 126 days after operation	Animals surviving
Low iodine plus supplemental iodine	Intact Sham operation	5	225·8 ± 6·1*	333·7 ± 9·2	4
	Thyroidectomized	5	247·2 ± 6·7	261·7 ± 8·2	4
Low iodine	Intact Sham operation	5	218·4 ± 3·3	326·4 ± 5·5	5
	Thyroidectomized	5	228·0 ± 8·3	178·3 ± 7·1	3

* Probable error of the mean.

conclusion of the experiment, of those animals which received the larger amounts of iodine, four of the control animals and four thyroidectomized animals were alive. Of those animals given the reduced amounts of iodine, all five of the controls and three of the thyroidectomized animals were alive.

In computing the changes or increases of the weights of the animals during the postoperative period, the average preoperative weight of those animals which were alive when the experiment was terminated was determined. These average weights do not appear in the table. Of the control animals, on which a sham operation had been performed, those receiving the higher levels of iodine gained 105·7 ± 11·8 g. during the postoperative period. Those which ate the diet with the smaller amounts of iodine gained 108·0 ± 6·4 g. Thus the daily iodine intake was without significant effect on the rate of growth of the control animals. Differences of growth rates, due apparently to the levels of iodine intake, were recorded, however, for thyroidectomized animals. Thyroidectomized animals which received the high levels of iodine daily gained an average of 25·2 ± 5·8 g., while those which received the lower amounts of iodine lost an average of 46·4 ± 13·2 g. A comparison of the average increase of the weight of one group with the marked average decrease of the weight of the other group yields a difference of 71·6 ± 14·4 g., a difference statistically significant.

Determinations of respiratory metabolism were recorded before the operation and at six times during the postoperative period. The preoperative determinations and three of the postoperative ones are included in Table 3. At the end of the preoperative period of 95 days there were no significant differences of the average caloric output between those animals given high levels of iodine and those given low levels. The caloric output of the control animals, on which a sham operation had been performed, decreased slightly from preoperative levels during the 126-day postoperative period. The caloric output of control animals receiving the higher amounts of iodine decreased 5·1 ± 0·9 cal./hr./sq.m. of surface during that period, while the output of those on the lower levels of iodine decreased 9·2 ± 3·1 cal. from

Table 3. *Effect of intake of iodine on caloric output of thyroidectomized and non-thyroidectomized animals*

Average calories per hour per square metre of surface area

Diet	Before operation	Animals	Condition of animals	After operation: 16 days	Animals	69 days	Animals	126 days	Animals
Low iodine plus supplemental iodine	48·6 ± 0·9*	10	Controls	46·9 ± 1·6	5	44·7 ± 0·9	5	43·5 ± 0·2	3
			Thyroidectomized	39·1 ± 0·5	5	30·7 ± 1·2	5	33·1 ± 1·5	4
Low iodine	49·1 ± 1·5	9	Controls	44·2 ± 1·6	4	39·9 ± 0·8	4	39·9 ± 2·7	4
			Thyroidectomized	34·1 ± 2·2	3	24·2 ± 1·7	3	26·7 ± 0·9	3

* Probable error of the mean.

the preoperative average caloric output. But on the 126th postoperative day there was no significant difference between the average caloric output of the control animals given high iodine (43·5 ± 0·2) and that of the control animals given low iodine (39·9 ± 2·7) (Table 3).

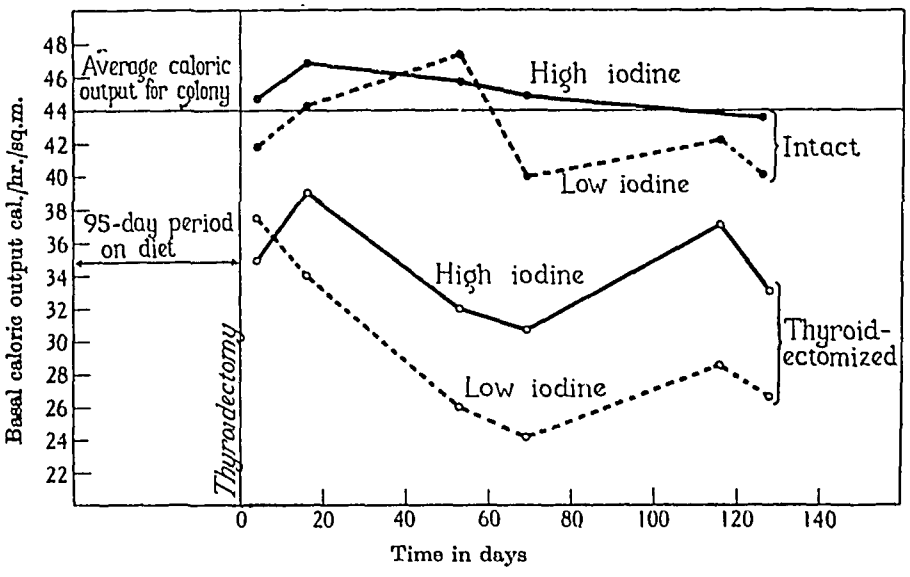


FIG. 1. Basal caloric output at six postoperative intervals of intact control animals, on which a sham operation had been performed, and thyroidectomized animals on the high and low levels of iodine intake.

Significant differences of the average caloric output with reference to the iodine intake occurred, however, among animals which had been thyroidectomized. Among such thyroidectomized animals given high levels of iodine, the caloric output on the 126th postoperative day was 15·5 ± 1·7 cal. less than the average preoperative determination for that group. Among such animals given low levels of iodine, the caloric output on the 126th postoperative day was 22·4 ± 1·7 cal. less than their average preoperative determinations. The difference between the average caloric

output of the thyroidectomized animals given high iodine and of those given the low amounts of iodine, on the 126th postoperative day, was $6{\cdot}4 \pm 1{\cdot}7$ cal./sq.m./hr. This difference is statistically significant (Table 3).

COMMENT

The data obtained in this study confirmed those reported previously [Chapman, 1941]. The influence apparently exerted by iodine on the body weight, during the growth period, and on the basal metabolism in thyroidectomized animals suggests that iodine may serve a physiological function similar to that attributed to thyroid-processed iodine. Abelin [1934] was among the first to produce substances, which had a thyroxine-like activity, by the *in vitro* iodination of protein. He concluded that the question of whether the thyroid cell possesses the exclusive ability to utilize iodine or whether this ability may be shared by other cells of the body remained unanswered. From our data it appears that perhaps somatic cells, as well as those comprising the thyroid gland, may utilize iodine and induce thereby a thyroxine-like effect.

Curtis [Personal communication to the authors] has demonstrated the presence of a high concentration of iodine in primitive organisms which do not possess a localized thyroid organ. He showed further that, among higher animals which do possess a specialized tissue for the efficient processing of iodine, the concentration of iodine in tissues exclusive of the thyroid is relatively low. Gorbman & Creaser [1942] have demonstrated that the endostyle of larval lampreys, a primitive anlage of thyroid tissue, selectively concentrates iodine.

It may be that body tissues, other than the thyroid, have retained a primitive ability to utilize iodine, although in a much less efficient manner than does the specialized thyroid tissue. The maintenance of the experimental or clinical subject in which thyroid function is lacking at a metabolic level which is compatible with life may depend on this primitive mechanism still resident in some cells.*

SUMMARY

Normal young rats were fed a low-iodine diet for 95 days. Half of them received $1{\cdot}5\mu$g. of iodine as potassium iodide in each cubic centimetre of their distilled drinking water. On the 95th day those animals which received the supplemental iodine were not statistically heavier than those which received the low daily amounts of iodine.

Half of all animals receiving low amounts of iodine, and half of those receiving the added amounts of iodine, were thyroidectomized on the 95th day. All others served as controls, on which sham operations were performed. The period of observation extended for 126 days after the operation. There were no statistically significant differences between the average weights of the control animals on the higher levels of iodine intake and those receiving the lower amounts. In intact animals the amount of iodine taken daily did not influence the rate of growth. A significant

* After this manuscript had been prepared for publication the interesting paper by Morton, Chaikoff, Reinhardt & Anderson [1943] appeared. Using radioactive iodine, these authors concluded that thyroxine may be produced in the thyroidectomized animal, and thus supported the conclusions of one of us [Chapman, 1941]. Their results are in accord with the conclusions reached in the present study.

difference, however, was observed in the average weight, on the 126th postoperative day, between the thyroidectomized animals given larger daily portions of iodine and those fed only the low-iodine diet. Thyroidectomized animals given supplemental iodine gained $25{\cdot}2 \pm 5{\cdot}8$ g., while the unsupplemented thyroidectomized animals lost, during the same period, $46{\cdot}4 \pm 13{\cdot}2$ g.

The average caloric outputs, calculated from oxygen consumption, of both control groups, receiving either the high or the low amount of iodine, were lower on the 126th postoperative day than before the operation. The averages, however, were statistically alike. When caloric outputs obtained from thyroidectomized animals were compared, it was evident that higher caloric values obtained in animals which received the larger portions of iodine.

The conclusion was again reached from data assembled on the weights of the body and from metabolic rates that iodine may be utilized by the body in the absence of the thyroid gland.

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ATELEIOTIC DWARFISM WITH NORMAL SEXUAL FUNCTION: A RESULT OF HYPOPITUITARISM

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(*Received 8 August 1943*)

The general classification of dwarfs is in many ways unsatisfactory. The group of ateleiotic dwarfs, as described by Hastings Gilford [1911], comprises some who are infantile, some progeric and some sexually mature. Rischbieth [1912] says that ateleiotics are usually infantile and sterile but that this is by no means invariable. Moreover, the condition is familial. After reviewing a number of different hypotheses that have been advanced to account for the occurrence of ateleiosis Rischbieth expresses the view that a defect of the pituitary is the most likely explanation, although any positive evidence on that score is lacking. There would seem, therefore, to be no distinction between ateleiosis and some forms of hypopituitary dwarfism.

Shelton [1938], in a discussion of pituitary dwarfism, draws attention to the fact that while most hypopituitary dwarfs show signs of general dysfunction of the gland and are sterile, some are fertile. He suggests the possibility that such persons might be found, at autopsy, to lack acidophil cells in the pars distalis. I have been unable to find any record of such a finding and for this reason believe the following case worthy of publication.

DESCRIPTION

Case report

A female dwarf aged 76 was found (1 November 1942) collapsed in her room. She was apparently a mental defective and lived in great squalor in a slum dwelling with some friends.

On inquiry, it was found that she had one illegitimate child, a woman aged 40, who was in a home for mental defectives. This woman was traced and found to be a high-grade mental defective of a happy disposition. Her height was exactly 5 ft. and she showed no apparent skeletal abnormality. She, like her mother, had some hair on the upper lip and a few hairs on the chin. Her eyebrows and head hair were dark and abundant. Her menstrual history was normal.

On admission to hospital the dwarf was cold, grey and almost pulseless. She had fever, and indefinite signs in the lungs. Urine normal. Blood W.R. negative. C.S.F. normal. B.P. 175/90. Died 13 November 1942.

Autopsy report 10 hr. after death

A poorly nourished old woman of spare build, 3 ft. 11 in. in height. Sabre-like deformity of tibiae with sharp anterior margins. Radial deviation of terminal phalanges of index fingers but no other skeletal abnormality. The long bones were slender and the epiphyses not enlarged. A little dark hair on the upper lip but no real hirsutism. Eyebrow hair abundant, with normal distribution. Head hair very thick. Death was due to a *Staphylococcus aureus* bronchopneumonia following coronary occlusion. Considerable coronary and cerebral atheroma. Some senile

atrophy of the brain; no lesions in the hypothalamic region. A large chronic gastric ulcer and a small lipoma in the wall of the jejunum were incidental findings. The uterus and adnexa were normal for a woman of this age.

Endocrine organs

The *pituitary body* weighed 0·31 g.; on gross section the only apparent abnormality was its small size, but histological study of sections showed extremely few alpha (acidophil) cells. The posterior lobe showed no abnormality. Sections were stained with Gallego's carbol-fuchsin orange G aniline blue and by various other polychrome staining methods. Unfortunately, the fixation, in formalin, was not perfect, and a proper differential cell count by the method of Rasmussen [1929] was not practicable. A general impression gathered from study of many horizontal serial sections through the gland may perhaps be strengthened by the result of a count of a total of 2000 cells from six different areas. This count gave results as follows: chromophobe cells 90·85%, alpha (acidophil) cells 0·2% and beta (basophil) cells 8·95%. In Rasmussen's series of non-pregnant women between the ages of 16 and 84 the percentage of alpha cells varied between 19 and 58. The alpha cells in the case now reported were not only few in number but poor in quality. They were small, measuring around 9 μ in diameter. The granules were scanty and irregularly grouped, and the cytoplasm of some of them was vacuolated, an unusual finding in alpha cells. These vacuolated cells tended to be larger than the others but none exceeded 11 μ in diameter. The diameter of the beta cells varied between 11 and 20 μ, the majority being about 14 μ in diameter. The cytoplasm of these cells was considerably vacuolated but not abnormally so for an aged woman. The chromophobe cells showed no abnormality, all being of the small type. A normal amount of colloid was present in the pars distalis. The pars intermedia was represented by simple clefts; wandering beta cells were present in small numbers and showed no abnormality.

The *pineal body* looked a little larger than normal, measuring 10 × 9 × 6 mm., but sections showed no abnormality.

The *thyroid gland* though small was symmetrical, and its size bore approximately the normal relationship to that of the larynx. Histologically the acini were very poorly formed, the majority being very small and irregular. The few larger acini were lined with flattened epithelium and deficient in colloid. There were no vascular changes and no abnormal collections of lymphocytes.

The *thymus* was entirely atrophied.

The *adrenals* together weighed 5·4 g. and were clearly small but in proportion to the size of the kidneys, each of which weighed 65 g. On gross examination no abnormality was noted. The cortex was not nodular. Histologically most of the cortical cells were small and dark staining; a few contained small vacuoles of lipid, most of which was anisotropic, crystalline and sudanophobe. The medulla showed no change.

DISCUSSION

The tibial deformity might possibly have been due to early rickets but there was no other sign to suggest this diagnosis, and in view of the findings in the pituitary gland it is clear that the dwarfism was of pituitary origin. The case would have been of greater interest if death had occurred before the menopause because at the age of 76

the genital organs are naturally atrophied and the thyroid gland may perhaps be expected to show senile involution. It would be unwise, in the absence of any physical indication of myxoedema, to claim that there was a lack of thyrotropic hormone.

The existence of a daughter of normal stature, albeit mentally defective, is proof of normal sexual function and presumably normal genital structure when the patient was aged 36. Her dwarfism was apparently of pituitary origin, and at autopsy her small pituitary gland was grossly deficient in alpha cells.

In thirty normal postmenopausal glands Biggart [1935] found an insignificant reduction of their alpha cells, 46% as against 50% in a series of ten normal preclimacteric glands. This shows that senile involution cannot be held responsible for the extreme reduction (0·2%) in alpha cells in this case.

In a strain of mice investigated by Snell [1929], Smith & Macdowell [1930, 1931], Kemp [1933, 1935, 1938], Kemp & Marx [1936, 1937], and De Beer & Gruneberg [1940], a rather similar condition has been found. These mice developed, as a mutation, a recessive Mendelian factor for dwarfism. They had complete, or almost complete, absence of acidophil pituitary cells, and biological examination of their pituitaries showed that they lacked the growth hormone but contained some gonadotropic hormone, although they were sterile. The reproductive organs of these mice, especially the males, were further developed than those of hypophysectomized rats and pituitary transplants readily rendered them fertile. In these mice there was a deficiency of growth hormone and to a lesser degree of gonadotropic hormones, associated with a gross deficiency of alpha cells.

It is generally conceded that the alpha cells produce the growth hormone, and it seems that the beta cells are concerned with the production of gonadotropic hormones. Severinghaus [1938] has shown that there is an increase in the number of beta cells in castrated animals and suggests that the increased gonad-stimulating potency of the anterior hypophysis of these animals is due to gonadotropic activity of the beta cells; but he believes, on the basis of other experimental work, that the alpha cells are also involved in the gonadal relationship. The observations on Snell's mice support this view, while the findings in the human case reported in this paper show that there may be a very great deficiency of alpha cells, enough to cause dwarfism, without precluding the possibility of pregnancy.

SUMMARY

The case is reported of a dwarf senile woman who had a child at the age of 36. At autopsy her pituitary gland showed a deficiency of acidophil cells.

My grateful thanks are due to Dr N. G. B. McLetchie who has examined some of my sections of the pituitary after staining them by some methods that he is now publishing [1944]. Some of his observations on the histology are incorporated in my report. My thanks are also due to Professor C. Bruce Perry for the use of his clinical notes and to Dr Datta for allowing me to see the patient's daughter, who is an inmate of the Stapleton Institution.

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OBSERVATIONS ON THE OESTROGENIC POTENCY OF STILBOESTROL IN THE GUINEA-PIG

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(*Received 29 October 1943*)

Included in the uterine changes which occur in the guinea-pig during normal oestrus is a relative hyperaemia at the antimesometrial border of the bicornate uterus [Bacsich & Wyburn, 1940], and as a similar vascular response has been induced in spayed animals by treatment with oestradiol monobenzoate [Bacsich & Wyburn, 1941] it can be considered as a characteristic of oestrogenic activity. In this work it was also shown that, while vaginal opening, cornification, increase in uterine weight, and all the so far recognized histological features of a typical heat response of the uterus can be elicited in practically 100% of spayed guinea-pigs by a comparatively simple administration of oestradiol monobenzoate, for the successful display of the cyclic vascular change of the uterus with any reasonable certainty, a more specialized treatment, including preliminary priming with small doses, is necessary. Moreover, it was demonstrated that the antimesometrial hyperaemia is an oestrogenic response of the adult uterus and cannot be induced in immature guinea-pigs with either oestrogenic [Bacsich & Wyburn, 1942*a*] or gonadotropic [Bacsich & Wyburn, 1942*b*] stimulation. This relative hyperaemia involving the antimesometrial part of the uterus seems to us, therefore, to provide a sensitive and specific test of oestrogenic activity, and as such has been utilized in these experiments as a method of biological assay of stilboestrol and the results compared with those previously obtained with oestradiol monobenzoate.

MATERIAL AND METHODS

Twenty-five adult female guinea-pigs were observed for a period and then ovariectomized by the standard method through lateral incisions. After an interval of from 4 to 5 weeks subcutaneous injections of stilboestrol dipropionate* (neo-Oestranol II, Crookes) were given. Vaginal smears were examined at regular intervals after the vaginae opened and animals were subsequently killed. At post-mortem examination the absence of ovarian tissue was verified and the uterus was observed and removed. Thick (100 μ) sections of the middle portion of the uterus were cut and prepared by the benzidine method [Bacsich & Wyburn, 1940] for the study of the vascular architecture. Thin (10 μ) sections were also cut and stained with haemalum and eosin for the examination of the histological changes in the endometrium.

DESCRIPTION

Group I. A preliminary experiment was carried out on four animals. They were primed with a daily injection of 0·0005 mg. of stilboestrol for 10 days. Results were completely negative as the vaginae remained firmly closed. The method of priming and the dose given were based on our previous work with oestradiol monobenzoate

* Referred to as stilboestrol hereafter in description of experiments and results.

when, following a daily priming dose of 0·0005 mg. of oestradiol monobenzoate (5 i.b.u.), the vaginae opened on the 5th or 6th day [Bacsich & Wyburn, 1941].

Group II. In a second series, six animals were primed with daily injections of 0·001 mg. of stilboestrol and in three animals the vaginae opened on the 8th day. These three animals were then given an injection of 0·5 mg. of stilboestrol on two consecutive days. Vaginal smears were examined at daily intervals from the 8th to the 11th day when the animals were killed. Cornification of the smears was present in each case on the 8th day and, after the larger doses, was complete. *Autopsy:* Uteri were enlarged and hyperaemic with apparent segmental distribution of blood vessels. *Histological examination:* In all three animals there was evidence of good uterine response such as endometrial proliferation, enlargement of glands, etc. *Vascularity:* There was a somewhat increased, but evenly distributed, generalized vascularity (Plate 1, fig. 1), although in animal 7 there was evidence of attempted antimesometrial hyperaemia, which was, however, confined to but a few sections of the sexually active part of the uterus (Plate 1, fig. 2).

Group III. Three animals were primed with 0·0025 mg. of stilboestrol per day and the vaginae opened on the 6th day. 0·5 mg. of stilboestrol was injected on the 7th and 8th days. The early vaginal smears (6th day) showed a greater degree of cornification than in group II. *Autopsy:* Uteri were enlarged and hyperaemic. *Histological examination:* Good endometrial response and in one case (animal 11) the uterine glands were particularly branched and tortuous. *Vascularity:* There was a uniform increased vascularity, but no indication of antimesometrial hyperaemia.

Group IV. Seven animals were primed with 0·0025 mg. of stilboestrol on six consecutive days until the vaginae opened. Thereafter daily injections of 0·5 mg. of stilboestrol were given for 6 days. The animals were killed on the 12th day. Early smears showed cornification, which after the first large dose was 100%. After the second and third doses deep epithelial cells and leucocytes appeared, and finally the smears became almost completely leucocytic. *Autopsy:* Uteri much enlarged and presented a cyanotic oedematous appearance. *Histological examination:* All animals had a particularly vigorous endometrial response. Endometrium was greatly thickened and oedematous, uterine epithelium increased in height, uterine glands were enlarged, tortuous and occasionally distended with secretion. *Vascularity:* Five of the animals showed an increased, but evenly distributed, vascularity throughout the sexually active middle portion of the uterus. Animal 14 had a more irregular vascular response: in some sections there were very hyperaemic or even haemorrhagic areas to one or other side, or involving the antimesometrial half, but there was no indication of a uniform antimesometrial hyperaemia (Plate 1, fig. 3). In animal 15 the sections of the uterus displayed well-marked antimesometrial hyperaemia throughout the greater part of the middle portion of the uterus, which contrasted with a relative avascularity at the antimesometrial pole itself (Plate 1, fig. 4).

Group V. Five animals were primed for 6 days with 0·0025 mg. of stilboestrol. Vaginae opened on 6th day. The animals were then given 1 mg. of stilboestrol for 5 days and a few hours after the last injection were given 0·2 mg. of progesterone. Vaginal smears exhibited changes similar to those of animals in group IV. *Autopsy:* Uteri were again enlarged and cyanotic. *Histological examination:* The uteri of all the animals showed a good endometrial response, more or less similar to that obtained

in animals of the previous group, but on a rather reduced scale. *Vascularity:* In one animal (no. 22) there was increased, but evenly distributed, vascularity. Animal 25 exhibited some occasional portions with good antimesometrial hyperaemia, but elsewhere the changes were similar to those in animal 22. In the remaining three animals (nos. 21, 23, 24) there was an antimesometrial hyperaemia involving the sexually active middle portion of the uterine horns. As with the endometrial changes, the vascular response was less (Plate 1, figs. 5, 6) than in animals 14 and 15, and more akin in its moderate degree to the appearance in normal oestrus.

DISCUSSION

Spayed guinea-pigs were injected with stilboestrol, and, using the vascular response as the method of biological assay, the results were compared with those obtained in a former work [Bacsich & Wyburn, 1941] after injections of oestradiol monobenzoate. The methods employed therefore follow closely those adopted in the experiments with oestradiol monobenzoate.

Dosage. It has been shown in a series of comparative studies by a variety of authors that stilboestrol is more effective orally than any of the natural oestrogens, but there is no unanimous verdict on the relative potencies following subcutaneous injection. Shorr, Robinson & Papanicolaou [1939] state that stilboestrol is only one-third to one-quarter as effective as oestradiol monobenzoate parenterally, and Emmens [1939] working with mice arrives at approximately the same conclusion. Kreitmar & Sieckmann [1939] find a biological equivalence for stilboestrol and oestradiol monobenzoate in the rat. The figures given by Sondern & Sealey [1940], in a publication dealing with the doses of oestrogenic materials to which castrated mice or rats respond, show that parenterally oestradiol monobenzoate is four times more effective than stilboestrol in mice, while in rats the two substances are equally active. Sealey & Sondern [1941] assayed stilboestrol on immature rats by a divided-dose technique and found stilboestrol four times more effective on the uterus and vagina than oestradiol: this they impute to the cumulative effect of stilboestrol. Matthews, Schwabe & Emery [1942], from extensive tests on castrate female rats, conclude that an alkaline aqueous solution of stilboestrol is thirty times more potent than an aqueous solution of oestrone, while Lee, Robbins & Chen [1942] using the response of the vaginal introitus of immature rats as their criterion find stilboestrol to be approximately twelve times as active as oestrone when administered orally and thirty-two times as active when injected subcutaneously. Morrell & Hart [1941], on the other hand, on the basis of the cornification of vaginal smears, give the ratio of oestrogenic activity of stilboestrol and oestrone in castrate female rats as 5:1. The discrepancy in the results obtained in these experiments in different animals implies, as suggested by Atkinson [1940], that no constant ratio of biological potency can be assigned to the different oestrogenic preparations but that this is to some extent a variable factor dependent upon the species of animal assayed and the method of assay. In the present experiment the minimum dose of stilboestrol necessary to ensure vaginal opening in 100% of cases was 0·0025 mg. daily for 6 days, whereas it has been found that 0·0005 mg. of oestradiol monobenzoate for 6 days sufficed. Therefore, so far as this particular test of biological activity is concerned, weight for weight, subcutaneous injection of stilboestrol is about one-fifth as

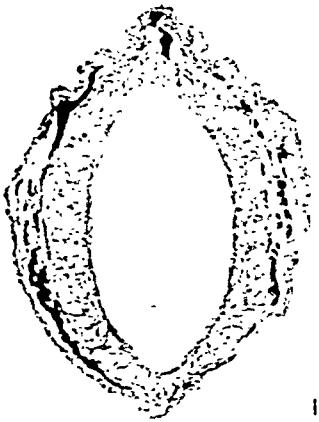
effective as oestradiol monobenzoate in the guinea-pig. The degree of cornification of vaginal smears compares very favourably with that obtained from animals treated with oestradiol monobenzoate. In fact, cornification occurs earlier, and following injection of larger doses achieves an intensity which is a striking feature of the cell picture. It is now appreciated, however [Marrian & Parkes, 1930], that a display of cornified cells in the vaginal smear does not necessarily reflect the complete uterine response, and that methods of biological assay, dependent only on vaginal reaction (such as the Allan-Doisy test) for the standardization of stilboestrol, are no fair index of oestrogenic efficiency *in toto*.

In all the animals killed there is an increase in the size of the uterus with endometrial proliferation and generalized increased vascularity. Such evidences of oestrogenic response are now an accepted result of stilboestrol treatment and have been described by numerous authors including Dodds, Lawson & Noble [1938] for the rabbit, and Palmer & Zuckerman [1939] for the monkey. Of the six animals given a daily dose of 0·5 mg. of stilboestrol for 2 days after preliminary priming, five show a uniform vascular distribution throughout the uterine sections (Plate 1, fig. 1), and in only one animal (no. 7) is there any indication of an antimesometrial hyperaemia (Plate 1, fig. 2), and that only occurs in an odd section. In a former work [Bacsich & Wyburn, 1941] similar treatment with oestradiol monobenzoate elicited a very marked and more constant local vascular response.

In this earlier investigation, after six daily injections of 0·5 mg. of oestradiol monobenzoate all animals showed an emphatic localized vascularity of the antimesometrial end of the uterus, but of seven animals given the same treatment with stilboestrol (group IV) only two (nos. 14 and 15) could be accounted as positive results, and even these were in some ways atypical (Plate 1, figs. 3, 4).

In group V the uteri of three out of five animals (nos. 21, 23 and 24—given a total of over 5 mg. of stilboestrol) present a picture of the moderate degree of antimesometrial hyperaemia which is comparable with that obtained in over 70 % of cases after a total dosage of 1 mg. of oestradiol monobenzoate [Bacsich & Wyburn, 1941], and resembles the physiological reaction of the normal animal on heat. Obviously in this experiment the greater constancy of positive results can be attributed mainly to the increase in the amount of stilboestrol injected. On the other hand, as we have previously suggested [Bacsich & Wyburn, 1941], the toning down of a too vigorous vascular response to oestrogenic stimulation is probably effected by the administered progesterone.

It therefore seems that for the complete uterine response, which includes the cyclic vascular change, as also for the vaginal opening, in the guinea-pig the ratio of biological potency of stilboestrol and oestradiol monobenzoate is 1:5. It should be noted, however, that methods of assay based on vaginal cornification, or non-vascular endometrial changes of the uterus alone, would denote a 1:1 ratio of oestrogenic activity for the two substances.



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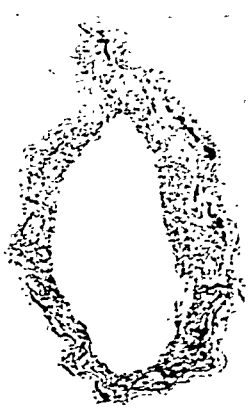
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SUMMARY

1. A test of stilboestrol dipropionate was carried out using the vascular response of the uterus of spayed guinea-pigs as the method of biological assay.

2. The results are compared with those of previous similar experiments with oestradiol monobenzoate.

3. Stilboestrol dipropionate can evoke the complete oestrogenic response in the spayed female guinea-pig including the characteristic vascular change.

4. The effective dose of stilboestrol dipropionate is five times greater than that of oestradiol monobenzoate.

We wish to acknowledge the helpful criticism of Professor D. M. Blair, and a grant from the Rankin Medical Research Fund in aid of the expenses. Neo-oestranol II was kindly supplied by Messrs Crookes Laboratories and the progesterone (Proluton) by Messrs Schering, Ltd.

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EXPLANATION OF PLATE 1

All illustrations are untouched microphotographs of cross-sections of the sexually active (middle) portion of the uteri of spayed guinea-pigs, stained by the benzidine reaction. Magnification × 18. Lower part of pictures represents the antimesometrial and the upper the mesometrial portions of the uteri.

FIG. 1. Group II. Animal 5. Somewhat increased, but evenly distributed, generalized vascularity.

FIG. 2. Group II. Animal 7. Attempted antimesometrial hyperaemia confined to a few sections of the sexually active part of uterus.

FIG. 3. Group IV. Animal 14. Irregular vascular response. Haemorrhagic areas involving the antimesometrial half of the uterus.

FIG. 4. Group IV. Animal 15. Well-marked antimesometrial hyperaemia of side walls, while antimesometrial pole is relatively avascular.

FIGS. 5, 6. Group V. Animals 21, 24. Vascular response approximating in its moderate degree the desired physiological ideal.

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